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Protection Against Ischemic Brain Injury by Inhibition of Mitochondrial Oxidative Stress

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Mitochondria are both targets and sources of oxidative stress. This dual relationship is particularly evident in experimental paradigms modeling ischemic brain injury. One mitochondrial metabolic enzyme that is particularly sensitive to oxidative inactivation is pyruvate dehydrogenase. This reaction is extremely important in the adult CNS that relies very heavily on carbohydrate metabolism, as it represents the sole bridge between anaerobic and aerobic metabolism. Oxidative injury to this enzyme and to other metabolic enzymes proximal to the electron transport chain may be responsible for the oxidized shift in cellular redox state that is observed during approximately the first hour of cerebral reperfusion. In addition to impairing cerebral energy metabolism, oxidative stress is a potent activator of apoptosis. The mechanisms responsible for this activation are poorly understood but likely involve the expression of p53 and possibly direct effects of reactive oxygen species on mitochondrial membrane proteins and lipids. Mitochondria also normally generate reactive oxygen species and contribute significantly to the elevated net production of these destructive agents during reperfusion. Approaches to inhibiting pathologic mitochondrial generation of reactive oxygen species include mild uncoupling, pharmacologic inhibition of the membrane permeability transition, and simply lowering the concentration of inspired oxygen. Antideath mitochondrial proteins of the Bcl-2 family also confer cellular resistance to oxidative stress, paradoxically through stimulation of mitochondrial free radical generation and secondary upregulation of antioxidant gene expression.

KEY WORDS: Superoxide; nitric oxide; peroxynitrite; pyruvate dehydrogenase; calcium; apoptosis.

MITOCHONDRIAL TARGETS OF OXIDATIVE STRESS

Several lines of evidence indicate that oxidative stress is a primary mediator of neurologic injury following cerebral ischemia. The extent of delayed neuronal death correlates well with prelethal markers of oxidative molecular alterations. Neuroprotection is observed following the use of antioxidants and inhibitors of free radical producing enzymes, e.g., nitric-oxide synthetase. In addition, neuroprotection is evident in genetic animal models where genes coding for enzymes that promote oxidative stress are knocked down or out, and where genes coding for antioxidant enzymes, e.g., superoxide dismutase (SOD) are overexpressed (see (Lewen *et al.*, 2000) for review).

Virtually every cellular and extracellular molecular component is potentially sensitive to damage caused by oxidative stress. Oxidative modification to DNA, RNA, proteins, lipids, and small metabolites occur during ischemia/reperfusion. Our research focuses on the mitochondrion and its components as both targets and mediators of oxidative reperfusion injury (Fiskum *et al.*, 1999; Murphy *et al.*, 1999). From both in vitro studies with

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neural cells (Myers *et al.*, 1995) and animal models of global cerebral ischemia (Liu *et al.*, 1998), we conclude that mitochondrial energy metabolism is extremely sensitive to impairment by reactive oxygen and nitrogen species (ROS/RNS) and that mitochondrial oxidative stress limits metabolic recovery and promotes the intrinsic pathway of apoptosis.

One hypothesis we are testing is that during reperfusion, pyruvate dehydrogenase (PDH) is oxidatively modified and inactivated (Bogaert *et al.*, 2000; Rosenthal *et al.*, 1992), resulting in impaired oxidative energy metabolism and exacerbation of postischemic brain lactic acidosis (Rosenthal *et al.*, 1992). PDH enzyme activity is lost when purified enzyme is exposed to systems that generate hydroxyl radicals (Bogaert *et al.*, 1994) or peroxynitrite (E. Martin, unpublished), two ROS/RNS species strongly implicated in reperfusion brain injury. In addition to inactivation of PDH, the activity of the electron transport chain Complex I (NADH-CoQ oxidoreductase) is also depressed during reperfusion (Almeida *et al.*, 1995), which could be particularly important since this complex is normally the rate-limiting step of the electron transport chain (Davey *et al.*, 1997). The relative importance of damage to components of the electron transport chain compared to upstream metabolic enzymes, e.g., PDH, is at this juncture unknown. However, decreased production of NADH by PDH and TCA cycle dehydrogenases may be responsible for the hyperoxidized redox state of NAD(H) and components of the mitochondrial electron transport chain that occurs during the first hour of reperfusion after global cerebral ischemia (Rosenthal *et al.*, 1995). If the electron transport chain was metabolically rate limiting during reperfusion, the NAD(H) redox state should be relatively reduced rather than oxidized. Postischemic oxidation of NAD(H) may therefore constitute an important clue for the identification of the most important metabolic targets of reperfusion injury.

Other possible explanations for the effect of ischemia/reperfusion on NAD(H) redox state include depletion of pyridine nucleotides through PARP activation (Wang *et al.*, 2002), and release of mitochondrial NAD(H) and NADP(H) from the mitochondrial matrix into the cytosol, e.g., what occurs following activation of the mitochondrial membrane permeability transition (MPT) (Chinopoulos *et al.*, 2003). In addition to inhibiting PDH and other mitochondrial enzyme activities, ROS/RNS are potent activators of both PARP and the MPT(Kowaltowski *et al.*, 2000; Prabhakaran *et al.*, 2004). Moreover, the metabolism of H_2O_2 and other peroxides via the glutathione peroxidase/reductase system can contribute to the oxidative shift in pyridine nucleotide redox state. Irrespective of the mechanism by which cerebral reperfusion causes this shift in redox state, the associated decrease in reducing power could limit detoxification of peroxides and maintenance of reduced protein sulfhydryl groups, thereby contributing to the prolonged oxidative stress characteristic of reperfusion tissue injury.

While oxidative damage to cerebral energy metabolism is a critical contributor to delayed, necrotic neuronal death, oxidative stress is also a powerful initiator of apoptosis, which also contributes significantly to ischemic neural cell death (DeGracia *et al.*, 2002; Hou and MacManus, 2002). The mechanism by which oxidative stress promotes apoptosis is far from understood. Possible mechanisms include increased expression of p53, a redox-sensitive transcriptional activator of several proapoptotic genes that also directly induces release of mitochondrial cytochrome *c* (CytC) through its interaction with the antiapoptotic mitochondrial protein Bcl-X_L (Chipuk *et al.*, 2003; Miller *et al.*, 2000; Soengas *et al.*, 1999). Reactive oxygen and nitrogen species can also induce the release of CytC from mitochondria through promotion of the MPT (Kowaltowski *et al.*, 2000; Borutaite *et al.*, 1999), although this event is more likely to cause necrosis due to the devastating effects of the MPT on mitochondrial energy metabolism. Oxidative alterations to mitochondrial membrane lipids or apoptotic proteins might also promote the release of CytC and other proapoptotic mitochondrial proteins through both MPT-dependent and independent mechanisms.

NEUROPROTECTION BY AVOIDING HYPEROXIA DURING CEREBRAL REPERFUSION

Intracellular conditions that exist early during reperfusion, e.g., low pH and high [Ca^{2+}], can promote the generation of ROS by mitochondria and other sources (Fiskum, 1997). Microdialysis measurements demonstrate high levels of hydroxyl radical production during the first 30–45 min of reperfusion (Piantadosi and Zhang, 1996). During this same period, hyperoxia exacerbates the oxidized shift in mitochondrial redox state and delays recovery of evoked potentials compared to what is observed with normoxic animals (Feng *et al.*, 1998). Several other studies have compared hyperoxic to normoxic reperfusion using histopathology as the outcome measure. Halsey implanted O_2 electrodes in the brains of rats before subjecting them to a 20 min global ischemic insult and found a positive correlation between reoxygenation level and severity of neuronal damage (Halsey *et al.*, 1991). Gerbils treated with 100% O_2 after 15 min bilateral carotid occlusion sustained increased white matter damage (Mickel *et al.*, 1990). A study using 15 min of cardiac arrest in

dogs followed by hyperoxic resuscitation found a significant increase in the total number of injured neurons in the brain stem and spinal cord within 1 h of resuscitation (Marsala *et al.*, 1992). Our preliminary results using a 10 min canine cardiac arrest model and sterologic cell counting indicate a significant reduction in hippocampal neuronal death using normoxic compared to hyperoxic resuscitation (V. Vereczki, unpublished). In contrast, Agardh used a rat model of transient global ischemia and failed to demonstrate differences in 7 day neuronal damage after resuscitation with 100% O_2 compared to normoxia or hypoxia (Agardh *et al.*, 1991). Lipinski *et al.* also found no difference in hippocampal neuronal death 72 h following cardiac arrest in rats ventilated on 100 or 21% O_2 (Lipinski *et al.*, 1999). This model is, however, significantly different from the canine cardiac arrest model or from most human cardiac arrest scenarios as the animals experience severe hypoxia prior to cardiac arrest.

The few reported comparisons of neurologic outcome following hyperoxic and normoxic reperfusion strongly suggest that hyperoxic resuscitation is detrimental. Using a 9 min canine cardiac arrest model, Zwemer found that resuscitation with 100%-inspired O_2 resulted in worsened 12 and 24 h neurologic outcome when compared to animals receiving 21% O_2 (Zwemer *et al.*, 1994). This difference was eliminated when animals were pretreated with an antioxidant prior to the arrest and hyperoxic resuscitation. In our canine experiments using 10 min cardiac arrest, neurologic impairment measured at 24 h was significantly worse in animals ventilated on 100% O_2 during and for 1 h after resuscitation than that exhibited by dogs resuscitated on 21% O_2 and subsequently ventilated on 21–30% O_2 to maintain normal PaO2 (Liu *et al.*, 1998). The one published negative study is the Lipinski report where no difference in 72 h neurologic impairment was observed following asphyxia-induced cardiac arrest in rats (Lipinski *et al.*, 1999). The only long-term outcome study focused on mortality and used the gerbil bilateral carotid occlusion model. Mickel and colleagues found that animals exposed to 100% O_2 for 3–6 h after 15 min global cerebral ischemia experienced a threefold increase in 14 day mortality compared with those allowed to breathe room air after ischemia (Mickel *et al.*, 1987).

MITOCHONDRIA AS SOURCES OF REACTIVE OXYGEN SPECIES

Superoxide is a normal byproduct of mitochondrial respiration and accounts for $\sim$1% of O_2 consumed by mitochondria. Because of its extremely high reactivity and short half-life, it normally dismutates to H_2O_2 either spontaneously or via catalysis by mitochondrial or cytosolic superoxide dismutases. While the metabolism of H_2O_2 via peroxidases can, under some circumstances, lead to oxidative stress due to an oxidized shift in cellular redox state, the primary toxicity of elevated superoxide and H_2O_2 production is exerted by other metabolites (Fig. 1). These products include the hydroxyl radical, generated by metal-catalyzed reduction of H_2O_2, and peroxynitrite, generated by the reaction of superoxide with nitric oxide. Both of these reactive agents are capable of oxidatively modifying proteins, lipids, RNA, and DNA. As there are no known enzymatic systems for detoxifying either hydroxyl radical or peroxynitrite, endogenous interventions are limited primarily to those that reduce the production of superoxide or nitric oxide, or that promote the nontoxic metabolism of H_2O_2 to H_2O via peroxidase activities. A number of additional exogenous antioxidant approaches are available, including the use of iron chelators, spin-traps, and other natural and artificial antioxidant compounds.

While the role of mitochondrial ROS production in ischemia/reperfusion injury is often touted as important, little direct evidence is available from in vivo experiments. Existing evidence is based on the effects of mitochondrial respiratory inhibitors or uncouplers on markers of oxidative injury. Additional support for a critical role of mitochondrial oxidative stress in acute neuronal cell death comes from in vitro experiments using cultured neurons and other neural cell lines exposed to hypoxia and glucose deprivation, or to toxic levels of excitatory neurotransmitters or their agonists. From these and more recent studies, it appears that initial entry of Ca^{2+} through glutamate receptors is accumulated into mitochondria, causing an increase in mitochondrial ROS production that then causes a secondary irreversible entry of Ca^{2+} through redox-sensitive transient receptor potential (Trp) channels (Aarts *et al.*, 2003).

One controversial topic in this field is the involvement of the MPT in Ca^{2+}-induced mitochondrial ROS production. MPT-mediated release of CytC can certainly stimulate mitochondrial generation of ROS by causing a reduced shift in mitochondrial redox sites associated with superoxide production. The MPT also causes a drop in mitochondrial membrane potential ($\Delta\Psi$) and a loss of mitochondrial pyridine nucleotides, both of which should depress mitochondrial generation of ROS. Recent work suggests, however, that even if mitochondrial NAD(H) were released into the cytosol in response to the MPT, the residual concentration in the mitochondrial matrix in equilibrium with the cytosolic pool could be sufficient to support substantial ROS production (Batandier *et al.*, 2004). The use of MPT inhibitors like cyclosporin A as neuroprotectants both in vivo and in vitro has met with

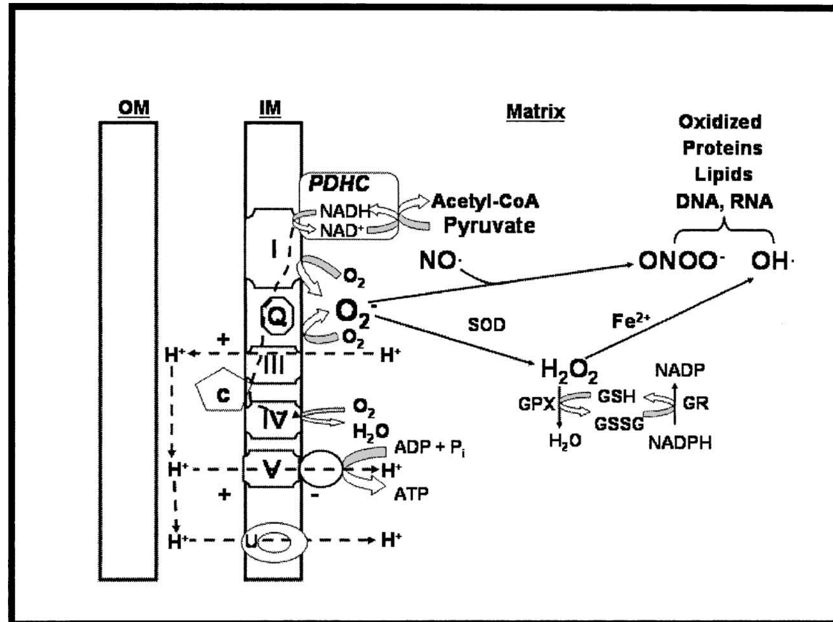

Fig. 1. Mitochondrial production and metabolism of reactive oxygen species. Superoxide (O_2^-) is produced at one or more sites within the electron transport chain and possibly via one or more matrix dehydrogenases. Superoxide is converted to hydrogen peroxide via superoxide dismutase (SOD), that is subsequently metabolized to water via the glutathione peroxidase (GPX)/glutathione reductase (GR) system. During abnormally high rates of hydrogen peroxide production and in the presence of transition metals, e.g., iron, the highly toxic hydroxyl radical is generated. Peroxynitrite can also be produced via the reaction of superoxide and nitric oxide. These metabolites can oxidize mitochondrial proteins, lipids, RNA, and DNA, contributing to oxidative stress and ultimately cell death.

mixed success (Domanska-Janik *et al.*, 2004; Kaminska *et al.*, 2001; Maciel *et al.*, 2003; Scheff and Sullivan, 1999; Uchino *et al.*, 2002). Evidence indicates that cyclosporin A is ineffective at blocking the MPT under many conditions and in certain cell types, including neurons (Fiskum *et al.*, 2003). Development of more broadly effective MPT inhibitors is therefore needed. One agent that exhibits superior inhibition of the MPT in brain mitochondria is 2-aminoethoxydiphenylborate (2-APB) (Chinopoulos *et al.*, 2003). This drug also inhibits capacitative Ca^{2+} entry associated with Trp channels and may therefore provide a multipotent approach to neuroprotection (Iwasaki *et al.*, 2001).

Several mechanism other than, or in addition to the MPT could stimulate mitochondrial ROS production during ischemia/reperfusion. Thermodynamically, any inhibition of electron flow distal to redox sites of superoxide production would promote these reactions. Thus, inhibition of electron transfer in Complex I distal to the putative iron sulfur site of superoxide generation, as occurs with rotenone, greatly stimulates ROS production with NADH-linked respiratory substrates. Inhibition at the distal points in the electron chain, as occurs with nitric oxide at Complex IV, stimulates ROS production at both Complex I and at the Coenzyme Q/Complex III redox site (Brown and Borutaite, 2001). A similar situation occurs when CytC is released during apoptosis via Bax-mediated formation of pores in the outer membrane (Starkov *et al.*, 2002). When electron flow is only partially inhibited, the redox-mediated stimulation of ROS production can be counteracted by mild uncoupling, either with exogenous uncoupling agents, e.g., FCCP, or via increased expression or activity of mitochondrial uncoupling proteins. Mitochondrial ROS production is extremely sensitive to inhibition by slight depolarization and oxidized shift in redox state at high membrane potentials (Starkov and Fiskum, 2003). Thus a drop in $\Delta\Psi$ of only 15 mV reduces the rate of NADH-linked, substrate-dependent ROS formation by 50% with little effect on ATP production. Mild uncoupling may therefore constitute an effective means of immediately reducing oxidative stress in acute CNS injury paradigms (Ferranti *et al.*, 2003; Kim-Han *et al.*, 2001).

In addition to inhibiting mitochondrial superoxide production, the net production of ROS can also be reduced by promoting its detoxification to H_2O_2 and then to

H_2O. Bcl-2, an antideath protein normally thought to act by binding to outer membrane pore-forming-proapoptotic proteins, e.g., Bax, also exhibits an indirect antioxidant activity that is apparent even at the mitochondrial level (Ellerby *et al.*, 1996). We demonstrated that the inhibition of pro-oxidant-induced MPT by Bcl-2 overexpression is due to increased resistance of pyridine nucleotides to oxidation rather than a direct effect on MPT proteins (Kowaltowski *et al.*, 2000). In an attempt to explain this phenomenon, we explored the effects of Bcl-2 and other antiapoptotic Bcl-2 family members on mitochondrial bioenergetics. Through careful calibration techniques, we found that mitochondria from Bcl-2-overexpressing cells do not exhibit higher membrane potential, in contrast to previous reports indicating a difference in $\Delta\Psi$. In agreement with other investigators, we found that overexpression of Bcl-2 was associated with an increase in basal mitochondrial ROS (H_2O_2) production. This counterintuitive phenotype was also observed in cells overexpressing Bcl-X_L and Mcl-1, two additional cytoprotective Bcl-2 family members (A. Kowaltowski, unpublished). Most importantly, when the overexpressing cells were treated for 48 h with low levels of uncoupler that eliminate the elevated level of mitochondrial ROS production, the cells lose their abnormally high peroxidase activity and their resistance to acute necrotic cell death caused by exposure to high concentrations of exogenous H_2O_2. It therefore appears that the antioxidant activity of at least three antiapoptotic Bcl-2 family members is similar to preconditioning paradigms where sublethal levels of stress cause upregulation of proteins that protect against normally lethal levels of stressful stimuli. While overexpression of Bcl-2 increases basal ROS production, this effect stimulates the expression of one or more antioxidant enzymes resulting in a net resistance to oxidative stress.

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