

Review Article

Novel mitochondrial targets for neuroprotection

Miguel A Perez-Pinzon^{1,5}, R Anne Stetler^{2,3,5} and Gary Fiskum^{4,5}

¹Department of Neurology, Cerebral Vascular Disease Research Center, University of Miami Miller School of Medicine, Miami, Florida, USA; ²Department of Neurology and Center of Cerebrovascular Disease Research, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA; ³Institute of Brain Sciences, Fudan University School of Medicine, Shanghai, China; ⁴Department of Anesthesiology and the Shock, Trauma, and Anesthesiology Research Center, University of Maryland School of Medicine, Baltimore, Maryland, USA

Mitochondrial dysfunction contributes to the pathophysiology of acute neurologic disorders and neurodegenerative diseases. Bioenergetic failure is the primary cause of acute neuronal necrosis, and involves excitotoxicity-associated mitochondrial Ca²⁺ overload, resulting in opening of the inner membrane permeability transition pore and inhibition of oxidative phosphorylation. Mitochondrial energy metabolism is also very sensitive to inhibition by reactive O₂ and nitrogen species, which modify many mitochondrial proteins, lipids, and DNA/RNA, thus impairing energy transduction and exacerbating free radical production. Oxidative stress and Ca²⁺-activated calpain protease activities also promote apoptosis and other forms of programmed cell death, primarily through modification of proteins and lipids present at the outer membrane, causing release of proapoptotic mitochondrial proteins, which initiate caspase-dependent and caspase-independent forms of cell death. This review focuses on three classifications of mitochondrial targets for neuroprotection. The first is mitochondrial quality control, maintained by the dynamic processes of mitochondrial fission and fusion and autophagy of abnormal mitochondria. The second includes targets amenable to ischemic preconditioning, e.g., electron transport chain components, ion channels, uncoupling proteins, and mitochondrial biogenesis. The third includes mitochondrial proteins and other molecules that defend against oxidative stress. Each class of targets exhibits excellent potential for translation to clinical neuroprotection.

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Introduction

Mitochondria are principal mediators of cell death that occurs during both central nervous system injury and in chronic neurodegenerative disorders; see Nagley *et al* (2010) and Schon and Przedborski (2011) for comprehensive reviews. Mitochondria are therefore important targets for neuroprotective interventions. The significance of mitochondrial

dysfunction was previously thought to be limited to effects on mitochondrial ATP production and therefore to necrotic cell death. We now understand that mitochondrial mechanisms of cell death include mitochondrial contribution to oxidative stress, apoptosis, and other forms of programmed cell death. Mild mitochondrial injury, with maintenance of near-normal cellular ATP, results in mainly programmed cell death, while more extensive injury that causes ATP depletion shifts the form of cell death toward necrosis (Ankarcrona *et al*, 1995).

Several fundamentally different, albeit interactive, mechanisms contribute to mitochondrial dysfunction and potentially cell death in both acute and chronic neurologic disorders. In neurons exposed to excitotoxic levels of the neurotransmitter glutamate, excessive mitochondrial accumulation of Ca²⁺ triggers several forms of mitochondrial injury, mediated

Correspondence: Dr G Fiskum, Department of Anesthesiology, University of Maryland School of Medicine, 685 West Baltimore Street, MSTF 5.34, Baltimore, MD 21201, USA.

E-mail: gfiskum@anes.umm.edu

⁵These authors contributed equally to this work.

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at least in part by Ca^{2+} -activated proteases (calpains) and phospholipases (Volbracht *et al*, 2001; Chiricozzi *et al*, 2010), and by opening of the inner membrane permeability transition pore (PTP) (Pivovarova and Andrews, 2010; Nicholls, 2009; Figure 1). A limited extent or duration of elevated Ca^{2+} may not result in the extent of bioenergetic impairment necessary for evoking acute cell death but can promote caspase-independent apoptosis through calpain-mediated mitochondrial release of apoptosis inducing factor (AIF) (Polster *et al*, 2005). Calpains can also cleave inactive BH3 domain-only proteins, forming active peptides, e.g., truncated Bid (tBid), which bind to proapoptotic and antiapoptotic proteins, e.g., Bax and Bcl2, resulting in outer membrane permeabilization and release of cytochrome c, AIF, and other proteins that trigger either caspase-dependent or caspase-independent programmed cell death (Krajewska *et al*, 2004; Cabon *et al*, 2012). A greater mitochondrial insult, which leads to delayed neuro-

nal Ca^{2+} deregulation, is more apt to induce rapid necrosis, particularly when PTP opening causes irreversible mitochondrial inner membrane depolarization and osmotic mitochondrial lysis (Pivovarova and Andrews, 2010).

Mitochondria are also highly sensitive targets of toxic reactive oxygen species (ROS) and reactive nitrogen species, respectively, generated by either intramitochondrial or extramitochondrial reactions (Figure 1). Mitochondrial sources of ROS include the electron transport chain, matrix dehydrogenases, free iron, and monoamine oxidases (Andreyev *et al*, 2005; Horowitz and Greenamyre, 2010). Numerous extramitochondrial sources of ROS and reactive nitrogen species include inducible NADPH oxidase, which is particularly important in the oxidative stress associated with cellular inflammatory activities (Sorce and Krause, 2009). Mitochondrial targets for damage by ROS and reactive nitrogen species include electron transport chain components, tricarboxylic

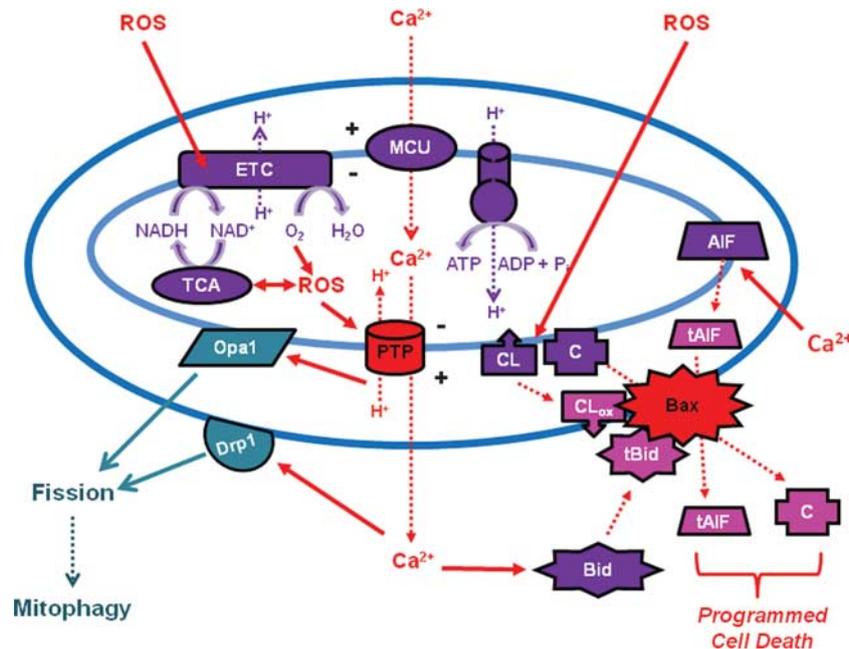


Figure 1 Mitochondrial mechanisms of neural cell death and targets for neuroprotection. The inner membrane electrical potential established by electron transport chain (ETC)-driven proton efflux is responsible both for ATP formation and for uptake of Ca^{2+} by the mitochondrial Ca^{2+} uniporter (MCU). Excessive Ca^{2+} uptake can activate the permeability transition pore (PTP), which releases accumulated Ca^{2+} and dissipates the proton gradient, thus uncoupling ATP synthesis from respiration. A critical decline in cellular ATP can cause acute, necrotic cell death. Elevated intracellular Ca^{2+} can also initiate mitochondria-dependent programmed cell death by activating calpain-mediated proteolysis of apoptosis inducing factor (AIF), normally bound to the inner membrane, and of Bid, normally located in the cytosol. Truncated Bid (tBid) can induce a conformation change in Bax, stimulating its oligomerization and megapore formation. Outer membrane megapores formed by Bax or Bak allow for release of intermembrane proteins, e.g., cytochrome c (C), and truncated AIF (tAIF), into the cytosol. These proteins trigger caspase-dependent and caspase-independent programmed cell death, respectively. Elevated Ca^{2+} promotes mitochondrial fission by at least two mechanisms that involve mitochondrial fission/fusion proteins. Membrane depolarization in response to Ca^{2+} -induced PTP opening stimulates proteolysis of Opa-1, an inner membrane fusion protein, which then promotes mitochondrial fragmentation. Extramitochondrial Ca^{2+} indirectly interacts with outer membrane dynamin-related protein 1 (Drp1), which also promotes fission. Reactive O_2 species (ROS) and nitrogen species produced by mitochondria and extramitochondrial sources target numerous mitochondrial molecules. Oxidative modification of proteins present in the ETC or the tricarboxylic acid (TCA) cycle inhibit aerobic energy metabolism. Oxidation of protein sulfhydryl groups also greatly increases sensitivity of PTP opening by Ca^{2+} . Peroxidation of cardiolipin (CL) decreases the amount of membrane bound cytochrome c and therefore increases the amount available for release to the cytosol. Oxidized cardiolipin also translocates to the outer membrane, where it enhances the ability of extramitochondrial Bax to bind and cause outer membrane pores to form.

acid cycle enzymes, the mitochondria-specific phospholipid, cardiolipin, and DNA (Andreyev *et al*, 2005; Sparvero *et al*, 2010; Chaturvedi and Beal, 2008). Oxidative stress also greatly increases the sensitivity of PTP opening by Ca^{2+} (Greco and Fiskum, 2010b). Due to the high rate of mitochondrial ROS production and the critical impact that oxidative damage to mitochondrial metabolism has on cell survival, mitochondria possess numerous systems for ROS detoxification and for inhibiting or reversing oxidative molecular modifications (Andreyev *et al*, 2005). These systems include low molecular weight antioxidants, e.g., glutathione, coenzyme Q, lipoic acid, and ascorbate. Antioxidant-related proteins include manganese superoxide dismutase (SOD2), glutathione peroxidase, peroxiredoxin, glutaredoxin, thioredoxin, glutathione reductase, and thioredoxin reductase. The reactions catalyzed by many of these enzymes depend on reducing power in the form of NADPH. Therefore, the mitochondrial redox state and the levels of mitochondrial enzymes that reduce NADP^+ , e.g., malic enzyme and NAD(P)H transhydrogenase, are equally important in defending against mitochondrial oxidative stress.

Mitochondrial damage can contribute to the vicious cycle of bioenergetic dysfunction and oxidative stress. Mitochondrial 'quality control' is maintained by the highly dynamic equilibrium between mitochondrial fusion and fission, segregating pathologic from healthy mitochondria, which are then targeted for disposal and recycling by mitochondrial autophagy (mitophagy) (Gottlieb and Carreira, 2010). This process together with mitochondrial biogenesis provides the highly efficient energy transducing machinery necessary for cell survival; therefore, impaired mitophagy or mitochondrial biogenesis can contribute to neuropathologies (Vosler *et al*, 2009).

Based on the extensive knowledge of mitochondrial mechanisms of acute and chronic neurodegeneration, mitochondria have been targeted by many experimental neuroprotective interventions. This review focuses on three relatively new classifications of mitochondrial targets for neuroprotection: mitochondrial dynamics, mitochondrial preconditioning, and mitochondrial antioxidants.

Mitochondrial dynamics

In nonpathogenic cells, mitochondria typically appear as a highly interconnected reticular network capable of mobilizing mitochondria to distant cellular regions, such as the neuronal synapse (Palmer *et al*, 2011). During cell division and throughout the lifespan of postmitotic cells, mitochondria undergo cycles of fusion and fission (1) to produce homogeneous daughter cells providing functional biochemical capabilities, (2) to isolate damaged mitochondrial subsets for targeted degradation (mitophagy), and (3)

to facilitate movement of mitochondria to various parts of the cell, including synapses (Palmer *et al*, 2011). Fusion and fission events are thus intimately entwined in both turnover and mobility. Although static imaging presents mitochondrial networks in most nonpathogenic quiescent cells as a reticular structure, mitochondria can be nonetheless undergoing transient ('kiss and run') fusion rather than remaining in a completely fused network (Twig *et al*, 2008). The rate of fission and fusion varies among cell types, and its significance is still widely unknown. However, as part of the flux between fission and fusion, mitochondrial biogenesis and mitophagy represent a delicate balance to maintain a consistent quantity of quality mitochondria, while specifically targeting damaged organelles for degradation without depleting cellular energetic supplies (Figure 2).

The molecular mechanisms of mitochondrial dynamics have become better understood in recent years, but remain elusive in understanding the full role of accessory proteins, many of which have complementary or overlapping functions. On the most basic level, fusion of mitochondria requires the two-step process of joining the double membrane (reviewed in Palmer *et al*, 2011). The primary proteins central to the initial fusion of the outer membrane are the GTPases mitofusin (Mfn)1 and Mfn2, which form homodimers or heterodimers *in trans* to allow for tethering of adjacent mitochondria. Concurrent to this, the mitochondrial phospholipase D hydrolyzes cardiolipin, which induces a structural bend and thus allows curvature of the fusing membranes (Choi *et al*, 2006). Several modulating proteins affecting fusion at the outer membrane have been identified, and include the proapoptotic molecules Bak and Bax. Fusion of the inner mitochondrial membrane centers on Opa-1, another dynamin-like GTPase protein. Opa-1 associates with the inner mitochondrial membrane, and is stabilized by Prohibitin and stomatin-like protein-2. Interestingly, Opa-1 differentially processed by changes in the subcellular environment, yielding distinct function in fusion/fission events. Opa-1 processing is sensitive to changes in the mitochondrial membrane potential (Ehse *et al*, 2009), where loss of membrane potential increases the processing of Opa-1 to shorter isoforms, which is then associated with increased fragmentation of the mitochondrial network (Palmer *et al*, 2011). This shift in mitochondrial morphology can be rescued by overexpression of long isoform of Opa-1. Alternatively, mitochondrial proteases may also target Opa-1 for cleavage. The specific regulation of fusion events at both the outer and inner membranes are still unclear, and are hampered by difficulty in model systems and molecular tools. Interestingly, Opa-1 has been implicated in antiapoptotic cristae remodeling, which appears to be distinct from its role in fusion (Ehse *et al*, 2009).

Fission of mitochondria can occur in the role of biogenesis, where intramitochondrial components are sorted and split into daughter mitochondria.

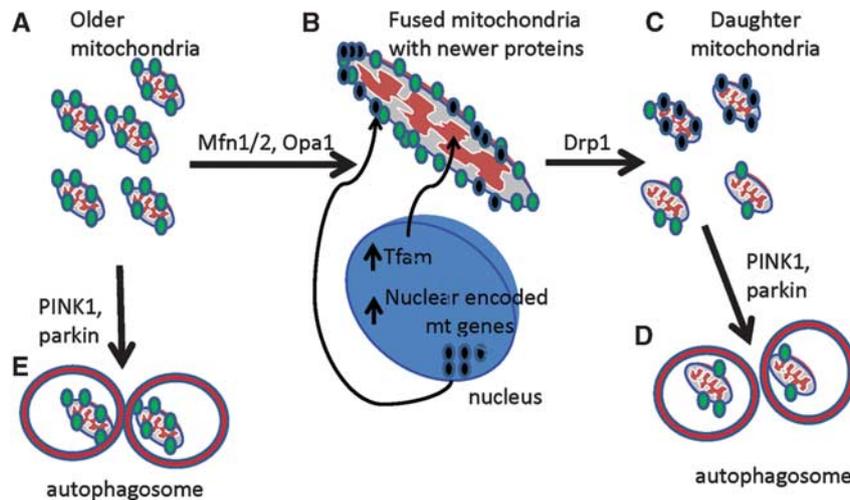


Figure 2 Biogenesis and mitophagy flux through fusion and fission. (A) Older mitochondria present in the cell may have loss of membrane potential (denoted by green dots, which could be due to older protein products of the electron transport chain). (B) These mitochondria can be fused and, in closer proximity to the nucleus, import newly synthesized nuclear-encoded gene products, such as electron transport chain subunits and the critical mitochondrial transcription factor TFAM. (C) The fused mitochondria then undergo fission to split into daughter mitochondria, which can produce a heterogeneous pool of mitochondria. Some mitochondria contain enough new material to maintain membrane potential, and are then kept to replenish the pool of functional quality mitochondria. However, some daughter mitochondria are not of high enough quality, and are thus targeted for mitophagy (D). (E) Damaged mitochondria can be targeted directly for mitophagy. Drp1, dynamin-related protein 1; PINK1, PTEN-induced putative kinase protein-1; Mfn1/2, mitofusins 1/2; TFAM, transcription factor A, mitochondrial.

However, fragmentation of mitochondria also precedes the selective targeting of mitochondria for mitophagy (Twig *et al*, 2008; Westermann, 2010). Dynamin-related protein 1 (Drp1) is a member of the dynamin family of GTPases, and is the major protein involved in the scission of membranes via translocation from the cytosol to the outer mitochondrial membrane for the formation of constricting rings (Palmer *et al*, 2011). Because Drp1 lacks a pleckstrin-homology domain, it requires a membrane receptor protein (e.g., Fis1) to associate with and polymerize at membrane. The Drp1 undergoes numerous posttranslational modifications, including phosphorylation, S-nitrosylation, ubiquitylation, and sumoylation (Cho *et al*, 2009; Palmer *et al*, 2011), which are not well characterized but are likely to reveal a highly controlled mechanism for responding to the cellular environment. Several of the identified modulators of Drp1 are Ca^{2+} or nitric oxide (NO) sensitive, and thus support the concept that fission is a functional response to a changing cell environment (Palmer *et al*, 2011).

The controlled regulation of fission and fusion events in the process of mitochondrial biogenesis is critical for the uniform distribution of mtDNA and the maintenance of consistent energetic production capacity. Although fusion and fission underlie the gross structural alterations required for mitochondrial biogenesis, a bigenomic upregulation of critical transcription factors and subsequent functional proteins is also necessary to maintain fidelity of biophysically and biochemically functional mitochondria (Figure 3). A cascade of nuclear transcription factors, including but not limited to peroxisome

proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) and nuclear respiratory factor-1 (NRF-1), are activated and upregulated, leading to the transcription of nuclear-encoded electron transport chain subunits and transcription factor A, mitochondrial (TFAM), a critical mitochondrial DNA transcription factor that is also involved in mtDNA replication. These proteins are specifically transported into the preexisting mitochondria and imported before fission and subsequent incorporation into the mitochondrial network (Ventura-Clapier *et al*, 2008). The resulting content of mtDNA as well as electron transport chain proteins per daughter mitochondria can vary significantly, and thus cannot be used as sole indicators of cellular mitochondrial content.

When newly formed daughter mitochondria have been incorporated into the mitochondrial network, mitochondria that have been damaged or that have lost membrane potential are specifically targeted for degradation via an autophagic-like process termed as mitophagy (Twig *et al*, 2008; Youle and Narendra, 2011). Originally discovered in yeast, the mitophagic process has recently been identified in mammalian cells, and is dependent on key proteins, such as the PINK1 (PTEN-induced putative kinase protein-1) and parkin, that are also particularly relevant to neurodegenerative diseases. However, much more detailed mechanistic work needs to be done to fully understand its role in physiologic and pathologic settings.

In recent years, abnormalities in mitochondrial fusion/fission, biogenesis, and mitophagy have been

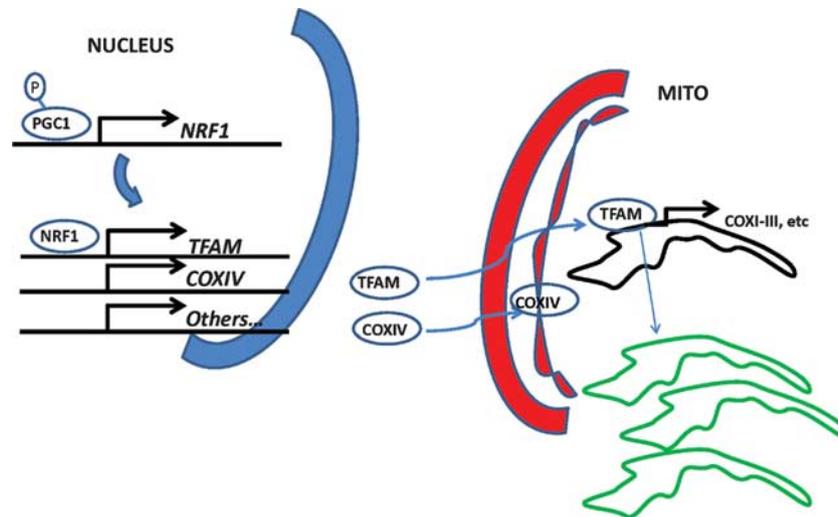


Figure 3 Bigenomic requirement of mitochondrial biogenesis. Critical transcription factors are activated (e.g., PGC-1 α) or upregulated (e.g., NRF-1), which leads to the upregulation of critical gene products for newly generated mitochondria, including the nuclear-encoded subunits of the electron transport chain, critical tRNAs, and TFAM, the major mitochondrial transcription factor necessary for new synthesis of mitochondrial-encoded proteins. TFAM also has a critical role in the replication of the mitochondrial genome. PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator 1-alpha; NRF-1, nuclear respiratory factor-1; TFAM, transcription factor A, mitochondrial.

identified in several neurodegenerative diseases, including Parkinson's, Alzheimer's, Huntington's and Charcot-Marie-Tooth diseases, as well as in nonneural injury states such as cardiac ischemia/reperfusion. Indeed, mutations of Mfn2 are associated with a subset of Charcot-Marie-Tooth (type 2A) (Zuchner *et al*, 2004) and Opa-1 in autosomal dominant optic atrophy (Alexander *et al*, 2000). Recently, PINK and parkin, proteins found to be mutated in a subset of PD patients, were recently identified as regulatory elements in directing mitophagy. Specifically, PINK and parkin appear to be highly involved in the induction of mitophagy. Disease-causing mutations lead to aberrant clearance of damaged mitochondria and defects in maintaining mitochondrial quality control (Youle and Narendra, 2011). In addition to disease-causing mutations, alterations in the posttranslational modifications of essential proteins in fission or fusion can also lead to neurodegeneration. For example, in a cellular model of Alzheimer's disease, β -amyloid protein increased mitochondrial fission in neurons. Mutation of the nitrosylation-sensitive cysteine residue of Drp1 effectively reduced the mitochondrial fragmentation as well as the neurotoxicity and synaptic damage due to β -amyloid exposure (Cho *et al*, 2009). Furthermore, aberrations in mitochondrial fission and fusion, mobility, and mitochondrial activity were observed in neurons cultured from transgenic mice overexpressing A β precursor protein (Calkins *et al*, 2011). In models of Huntington's disease, dominant-negative Drp1 decreased mitochondrial fragmentation and cell death in cells overexpressing mutant Huntington (mtHtt), the protein that underlies Huntington's disease (Wang *et al*, 2009). Addition-

ally, PGC-1 α transcriptional activity was significantly repressed by mtHtt overexpression, and subsequent overexpression of PGC-1 α was neuroprotective (Cui *et al*, 2006). Although PGC-1 α has many downstream transcriptional targets, mitochondrial biogenesis could be an important aspect to investigate.

The implications of mitochondrial dynamics in neural ischemia are still unknown, and only beginning to be investigated. Transient increases in mitochondrial proteins and mtDNA can be induced by ischemic injury, including neonatal hypoxia/ischemia (Rasbach and Schnellmann, 2007; Yin *et al*, 2008). Furthermore, modest changes in mitochondrial mobility and morphology were also observed in neuronal ischemia (Bertoni-Freddari *et al*, 2006; Yin *et al*, 2008; Valerio *et al*, 2011). The calcium- and oxidant-sensitive nature of several of the signaling molecules leading to mitochondrial dynamics underscore the need for further investigation of the role of mitochondrial responses as an organelle after neural ischemic injury.

Unfortunately, the technical assessments of mitochondrial dynamics are still lacking in reliability, applicability, and interpretation. Improvements in functional assays and end points as well as a critical sense of data interpretation are necessary to be able to accurately and consistently describe mitochondrial changes under different cellular environments. Changes in mitochondrial morphology have been observed in correlation with cell death stimuli, but most of the functional studies rely heavily on the knockdown or overexpression of singular proteins involved in fission/fusion, which can in and of itself render the cell more susceptible to cell stress. Furthermore, lack of experimental evidence of

manipulation of mitochondrial dynamics may also be difficult to interpret, as compensatory mechanisms are widespread throughout the system, or morphology may be left unchanged even in the face of altered *rates* of fission/fusion (Arnold *et al*, 2011). Combined with a high degree of heterogeneity present in the molecular makeup of individual mitochondria, it is critical for current investigations into mitochondrial dynamics to use a wide range of cellular, molecular, histological, and biochemical tools to fully explore the effects of changes in cell environments.

The concept of targeting an organelle as a therapeutic strategy remains a difficult process to imagine. However, the examples of aberrant fusion and clearance of mitochondria underlying pathology in models of Parkinson's disease, Alzheimer's disease and other neurodegenerative processes give merit to the idea of the organelle itself as a therapeutic or pathogenic target. Mitochondrial fusion and biogenesis have also been implicated in neuroprotective strategies against acute neural injury. Calpain inhibition provided neuroprotection against NMDA-induced neurotoxicity and was dependent on Opa-1, an enzyme critical for mitochondrial fusion (Jahani-Asl *et al*, 2011). Furthermore, induction of core elements of mitochondrial biogenesis have been observed using multiple preconditioning stimuli in neurons, such as sublethal hypoxia or hypoxia/ischemia (Gutsaeva *et al*, 2008), hyperbaric oxygen (Gutsaeva *et al*, 2006), and resveratrol (Dasgupta and Milbrandt, 2007; Biala *et al*, 2010). These stimuli have been well documented as neuroprotective strategies against acute brain injury, including cerebral ischemia and glutamate excitotoxicity. Thus, on a similar conceptual basis as cell restoration and phagocytosis, or organ transplant and resection, the targeted clearance and replacement of a damaged organelle could theoretically have a pluripotent impact on cellular (and hence, systemic) outcomes that is lacking by therapeutic strategies targeting a single molecule.

Ischemic preconditioning and mitochondria

Ischemic preconditioning (IPC) has gained attention as a robust neuroprotective mechanism against conditions of metabolic stress in different organs, including brain (Gidday, 2006). It is defined as the ability of either a brief ('sublethal') ischemic episode or mild stress imposed on a given organ, followed by a period of recovery, to increase an organ's resistance to injury. The cellular and subcellular mechanisms that support IPC are much better understood today, but still many of the signaling pathways and interaction among pathways remain undefined (see review Gidday, 2006). It is important to clarify that the main emphasis in this field is the elucidation of

the mechanisms by which IPC affords protection against cerebral ischemia, to discover potential therapies that can emulate this condition in the clinic.

One signaling pathway identified as key in the protection of brain mitochondria in preconditioning is protein kinase C (PKC). The role of PKC isozymes on cerebral ischemia was suggested by many studies in the past; however, development of novel pharmacologic tools that distinguish among the different isozymes offered new insights into specific PKC isozymes (Bright and Mochly-Rosen, 2005). Thus, the role of PKC isozymes on mitochondrial physiology/dysfunction has only recently emerged with new in-depth investigations in the brain.

A number of excellent reviews have been written describing the biochemistry of PKCs (Bright and Mochly-Rosen, 2005). In summary, PKC is a family of at least 12 serine/threonine kinases, which can be split into three broad categories: conventional, novel, and atypical (Bright and Mochly-Rosen, 2005), and they have multiple cellular roles. The conventional PKCs (α , β_1 , β_{II} , and γ) require Ca^{2+} , diacylglycerol, and phospholipid for activation. The novel PKC isozymes (δ , ϵ , η , and θ) lack the calcium-binding region, so these subtypes are not dependent on Ca^{2+} for activation but may be activated by Ca^{2+} indirectly with diacylglycerol. Activation of isozymes of the atypical PKC group (ζ , λ , μ , and ι) is also independent of Ca^{2+} . However, atypical PKC isozymes lack the Zn^{2+} finger region required for binding of diacylglycerol or phorbol ester. Instead, 3'-phosphoinositides may be the activators of atypical PKCs (Bright and Mochly-Rosen, 2005).

Among all these PKC isozymes, a number of groups have now confirmed that the novel PKC isozyme epsilon (ϵ PKC) is a key signaling pathway after IPC and has an important role in protection against lethal ischemia in brain (Bright and Mochly-Rosen, 2005). In brain, IPC leads to activation of ϵ PKC in hippocampus, the most vulnerable part of brain to global cerebral ischemia (Raval *et al*, 2007).

The ϵ PKC can protect brain mitochondria either by activation of other downstream signaling pathways or by its direct phosphorylation of mitochondrial targets. For example, downstream signaling pathways ϵ PKC include: the Src family of protein tyrosine kinases, the mitogen-activated protein kinase p38, the MAPK/ERK kinase MEK1/2, and the serine/threonine kinase Akt (Lange-Asschenfeldt *et al*, 2004; Greco *et al*, 2006). These signaling pathways (and potentially many others yet to be identified) evoke posttranslational modifications of existing proteins as well as transcriptional activation and *de-novo* protein synthesis that result in neuroprotection.

A more direct role of ϵ PKC on mitochondria leading to inhibition of apoptosis was previously observed by Baines *et al* (2002) who showed that transgenic mice expressing activated ϵ PKC formed ϵ PKC-ERK 'modules' in cardiac mitochondria. The ϵ PKC-ERK has been shown to directly target mitochondria

by phosphorylating subunit IV of the mitochondrial respiratory chain cytochrome *c* oxidase (COX) complex in cardiac myocytes (Guo *et al*, 2007).

Based on previous findings that showed that IPC was able to improve mitochondrial functions after cerebral ischemia (Perez-Pinzon *et al*, 2002), a link between ϵ PKC and mitochondrial neuroprotection was established. It was first established that ϵ PKC translocated early on to mitochondria after IPC and that one of the main targets was the mitochondrial K_{ATP}^+ channel (Raval *et al*, 2007; Figure 4). After IPC, selective inhibition of ϵ PKC activation prevented Kir6.2 phosphorylation, a specific subunit of the $m_{t}K_{ATP}^+$ channel, consistent with Kir6.2 as a phosphorylation target of ϵ PKC or its downstream effectors, and inhibition of this channel inhibited IPC-induced neuroprotection. In another study, ϵ PKC levels were found to increase in the hippocampal synaptosomal fraction 48 hours after IPC (Dave *et al*, 2008). Treatment with a specific ϵ PKC activating peptide 48 hours after IPC, increased the rate of oxygen consumption in the presence of substrates for complexes I, II, and IV (Dave *et al*, 2008). These increases in the rate of respiration were correlated with increased levels of serine and tyrosine phosphorylation of 18 kDa subunit of complex I, threonine phosphorylation of COX IV, increased mitochondrial membrane potential, and decreased H_2O_2 production. In addition, induction of *in-vitro* ischemia decreased mitochondrial cytochrome *c* release (Dave *et al*, 2008). These results suggest that

after IPC, ϵ PKC is readily available for activation in synaptosomal mitochondria on ischemia/reperfusion, in such a manner to increase mitochondrial respiration, reduce ROS production and cytochrome *c* release, hallmarks of reperfusion injury.

For many years, it was believed that one of the main causes of aging was the slow production of ROS over a long time, leading to a gradual decline in metabolism (Guarente, 2008). Although, the field of cellular aging has become much more complex, it is now accepted that the phenomenon of calorie restriction (CR) extends lifespan in many species and that this in part is due to mitochondrial modifications (Guarente, 2008). Calorie restriction is initiated by an increase in the ratio of cellular NAD^+ / $NADH$, which in turn activates a family of proteins called sirtuins (Guarente, 2008). Since CR is a type of metabolic stress that also targets mitochondria, CR proponents suggest that there may be similar pathways activated by both CR and IPC. Lending support to this hypothesis, anoxic preconditioning was found to decrease mitochondrial NAD^+ / $NADH$ ratio in hippocampal slices, as measured by spectrofluorometry (reviewed in Morris *et al*, 2011). This finding also suggests that cytosolic NAD^+ / $NADH$ was decreased after preconditioning. Since increases in cytosolic NAD^+ / $NADH$ ratio activate the sirtuin 1 (SIRT1) enzyme, these findings suggested that IPC might act by activating the SIRT1 pathway (Figure 4). This hypothesis was confirmed in recent studies in brain, demonstrating that showed that resveratrol, an

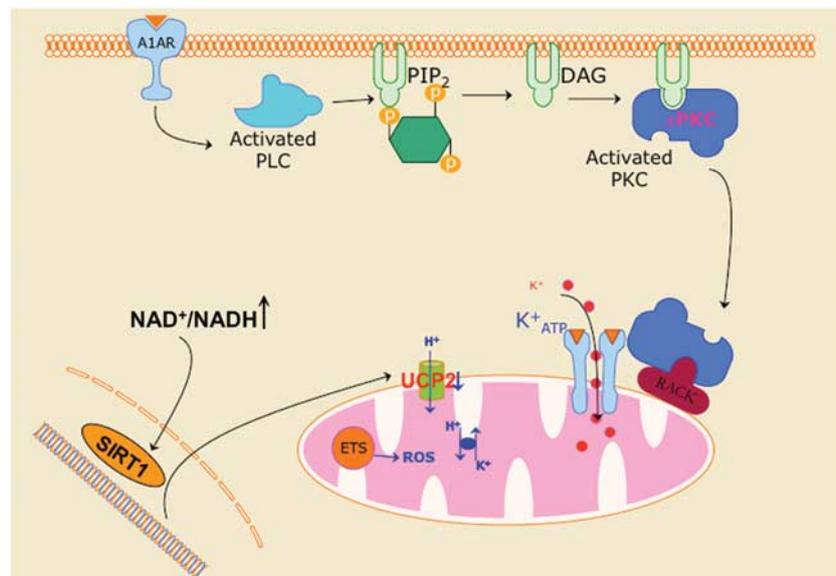


Figure 4 Mitochondrial targets of ischemic preconditioning. In brain, ischemic preconditioning (IPC) activates the adenosine A1 receptor (A1AR), which then activates phospholipase C (PLC). Phosphatidylinositol bis-phosphate is hydrolyzed by PLC forming diacylglycerol, which is a potent activator of protein kinase C isoforms, e.g., protein kinase C epsilon (ϵ PKC). ϵ PKC in turn binds to a mitochondrial receptor for activated C kinase (RACK), and activate the ATP-sensitive mitochondrial potassium channel ($m_{t}K_{ATP}^+$). Transmembrane cycling of potassium through the $m_{t}K_{ATP}^+$ channel and the K^+/H^+ antiporter may slightly depolarize the mitochondrial inner membrane and decrease production of reactive O_2 species (ROS) by the electron transport system (ETS). IPC also alters NAD^+ / $NADH$ levels, activating SIRT1, which leads to a decrease of the uncoupling protein 2 (UCP2).

activator of SIRT1, emulated IPC via the SIRT1 pathway and that blockade of SIRT1 abolished IPC (Raval *et al*, 2006; Della-Morte *et al*, 2009).

There are at least seven sirtuins (SIRT 1–7) identified in mammals. SIRT1 is the most extensively studied and has been implicated in lifespan extension of organisms including mammals (Guarente, 2008). SIRT1 exerts its effect by deacetylating histones (e.g., H1, H3, and H4), as well as nonhistone targets such as TAFI68 (TATA-box binding protein)-associated factor I, MEF2 (MADS box transcription enhancer factor 2), the transcription factor NF- κ B, tumor suppressor p53, a p53-related tumor suppressor p73, a DNA-repair factor, and inhibitor of Bax-mediated apoptosis Ku70 and FOXO (Forkhead box class O) transcription factors, among others (for specific references, see a recent review Morris *et al*, 2011).

One recent study showed that SIRT1 is present in the mitochondria where it may locally regulate mitochondrial biogenesis (Aquilano *et al*, 2010). However, the more conventionally recognized mitochondrial localized sirtuins are SIRT3, SIRT4, and SIRT5, which also have the ability to regulate mitochondrial dynamics and are implicated in CR, aging, and metabolic stress protection (Morris *et al*, 2011). Similarly to SIRT1, these mitochondrial sirtuins may augment mitochondrial activity and serve as novel targets for protection against cerebral ischemia. For a recent extensive review of these sirtuins, see Morris *et al* (2011).

A recent study by Dioum *et al* (2009) showed that SIRT1 deacetylated hypoxia inducible factor-2 α (HIF-2 α) that promoted expression of cytoprotective genes in culture mammalian cells. HIF-1 α and HIF-2 α are transcription factors that respond to low oxygen levels (Semenza, 2011). Under normal conditions, both HIFs are hydroxylated on proline residues, which then promote their degradation. Under hypoxic conditions, HIF proteins stabilize and accumulate leading to transcription of key cytoprotective pathways. HIF-1 α has been linked to hypoxic preconditioning in brain (Ran *et al*, 2005). SIRT1 activation of HIF-2 α was shown to enhance expression of mitochondrial ROS scavenger manganese SOD2 and erythropoietin (Dioum *et al*, 2009). Although the role of HIF-2 α has not been defined in IPC in brain, one of its downstream targets, erythropoietin, has been well characterized (Zhang *et al*, 2006). The IPC elevation of erythropoietin was sufficient to induce a preconditioned response (Meloni *et al*, 2006) and to protect rat CA1 neurons against ischemia (Zhang *et al*, 2006).

The transcriptional coactivator PGC-1 α is another well-known target of SIRT1. This pathway has been shown to mediate mitochondrial biogenesis by stimulating the expression of nuclear-coded mitochondrial proteins (Puigserver and Spiegelman, 2003). Recent studies suggest that PGC-1 α and SIRT1 may also reside in mitochondria and promote mitochondrial biogenesis in HeLa cells and mouse brains via interaction with TFAM (Aquilano *et al*, 2010).

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha has been associated with protection against oxidative stress. Enhanced levels of PGC-1 α protected hippocampal neurons against delayed cell death after transient ischemic episodes and decreased expression of manganese SOD and uncoupling protein 2 (UCP2) (Chen *et al*, 2010). Mice lacking PGC-1 α displayed enhanced hippocampal neurodegeneration in response to oxidative stress (St-Pierre *et al*, 2006) and exhibited decreased antioxidant expression after ischemia (Chen *et al*, 2010). Therefore, PGC-1 α has an important role in protection against ischemic death and ROS-induced oxidative stress.

SIRT1's neuroprotective effects against ischemia may also stem from direct modulation of mitochondrial activity. SIRT1 represses UCP2, a member of inner mitochondrial membrane proteins regulating proton electrochemical gradient, by binding directly to the UCP2 promoter (Bordone and Guarente, 2007; Guarente, 2008). A recent study showed that resveratrol treatment *in vivo* decreased UCP2 protein expression, which was blocked by an SIRT1 inhibitor (Della-Morte *et al*, 2009), suggesting that SIRT1 may regulate mitochondrial dynamics by influencing UCP2 expression. These multiple observations are compatible with SIRT1 acting through modulation of mitochondrial function.

In addition to the activation of SIRT1, resveratrol is also known to modulate the expression of several genes including COX-2 (cyclooxygenase-2) (inflammation), inducible nitric oxide synthase (NO production), endothelin-1 (vasoconstriction), and insulin-like growth factor binding protein (cell growth), among others (Morris *et al*, 2011). Recent studies showed that the signal transducers and activators of transcription 3 (STAT3) mediated COX-2 overexpression plays a key role in IPC-induced neuroprotection (Kim *et al*, 2008). STATs have been suggested to modulate mitochondrial function (Lin *et al*, 2011), and may be another signaling pathway involved in mitochondrial protection after IPC.

The STATs family consist of seven members 1, 2, 3, 4, 5A, 5B, and 6 (recently reviewed in Lin *et al*, 2011). The STAT activation can be proapoptotic or antiapoptotic. For example, STAT1 has been shown to induce apoptosis in cardiomyocytes, whereas STAT3 protects cardiomyocytes against ischemic injury (see Lin *et al*, 2011 for details). In brain, phosphorylation and nuclear translocation of STAT3 in response to IPC were observed in mixed cortical/glia cocultures (Kim *et al*, 2008). The STAT3 was found to exert neuroprotection through the transcriptional upregulation of COX-2. Also, another study showed that erythropoietin neuroprotection against CA1 pathology after cerebral ischemia was mediated by another STAT isozyme (i.e., STAT5; Zhang *et al*, 2007). Some of the mechanisms by which STATs regulate mitochondrial function include: (1) STAT1 activation leads to production of ROS, loss of

mitochondrial membrane potential, and upregulation of the mitochondrial adenine nucleotide translocator 3; (2) in contrast to STAT1, when STAT3 is knocked out or downregulated, impairments in complexes of the electron transport chain are observed; and (3) STAT3 appears to transcriptionally upregulate the activity of manganese SOD2 and deletion of STAT3 leads to increased production of ROS (reviewed in Lin *et al*, 2011).

Protection against mitochondrial oxidative stress

A wide range of experimental neuroprotective approaches have been used based on protection against mitochondrial oxidative stress. Strategic classifications include the following: (1) Inhibitors of ROS formation. (2) Exogenous antioxidants. (3) Agents that may increase reducing power necessary for ROS detoxification. (4) Stimulation of gene expression that increases mitochondrial antioxidant defenses.

There are two basic mechanisms by which the generation of superoxide by the mitochondrial electron transport chain or tricarboxylic acid cycle dehydrogenases can be regulated. The first is by controlling tissue O₂ levels and the second is controlling the redox state of redox sites that are responsible for superoxide production. Chance and coworkers showed decades ago that mitochondrial ROS production continues to increase with the ambient O₂ concentration up to 100% O₂ (Boveris and Chance, 1973). More recently, Brookes and colleagues showed that the K_m for O₂ for mitochondrial ROS production varies between 0.2 and 5.0 μmol/L, depending on the site of superoxide formation within the electron transport chain (Hoffman and Brookes, 2009). Since the K_m for O₂ for normal mitochondrial respiration is in the range of 0.1 to 1.0 μmol/L, both of these studies strongly suggest that the presence of abnormally high, hyperoxic, levels of O₂ in tissues can increase mitochondrial free radical production without significantly increasing respiration and therefore aerobic energy metabolism. An example of how avoiding hyperoxia can provide neuroprotection comes from studies comparing neurochemical and neurologic outcomes after experimental cardiac arrest, for animals artificially ventilated using either 100% O₂ or 21% to 30% O₂ during the first hour of reperfusion. Compared with animals receiving normoxic resuscitation, those receiving hyperoxic ventilation display elevated markers of oxidative stress, e.g., nitrotyrosine, impaired mitochondrial respiration, inhibited pyruvate dehydrogenase enzyme activity, decreased cerebral aerobic energy metabolism, increased hippocampal neuronal death, and worse neurologic outcome (Vereczki *et al*, 2005; Balan *et al*, 2006; Richards *et al*, 2007; Fiskum *et al*,

2008). These findings illustrate the concept that simple avoidance of unnecessarily high levels of O₂ can defend against oxidative stress-induced mitochondrial bioenergetic dysfunction and provide neuroprotection in a clinically relevant model of acute brain injury.

Another approach toward reducing mitochondrial superoxide production is shifting the redox state of mitochondrial electron transport chain components from a relatively reduced level, which promotes one electron transfer to O₂, to a more oxidized redox state, which decreases the thermodynamic force toward superoxide production. This oxidized shift in redox state is most commonly accomplished by the use of protonophore uncoupling molecules, which depolarize the mitochondrial inner membrane and inhibit oxidative stress and neuronal death in experimental models of cerebral ischemia, brain trauma, and Parkinson's disease (Korde *et al*, 2005). Thermodynamic inhibition of mitochondrial ROS production has also been used to explain how increased expression of mitochondrial 'UCP2' results in cytoprotection (Negre-Salvayre *et al*, 1997). While there are numerous examples of neuroprotection by either activation or overexpression of UCP2 (Paradis *et al*, 2003), the mechanism of protection by uncoupling and inhibition of mitochondrial superoxide production is not uniformly accepted (Cannon *et al*, 2006). The same lack of consensus applies to the mild uncoupling mechanism by which activators of the mitochondrial ATP-regulated potassium channel, e.g., diazoxide, provide neuroprotection (Xie *et al*, 2010).

Numerous exogenous antioxidants have shown neuroprotection in many different animal models of acute central nervous system injury and neurodegenerative diseases (Calabrese *et al*, 2009), and some of these appear to act either primarily or at least partially at the mitochondrial level (Chaturvedi and Beal, 2008; Yang *et al*, 2009). A few of the most widely studied of these agents include the hydrophobic natural compounds ubiquinone, α-tocopherol, and estrogen, which scavenge free radicals primarily at or within membranes. While progesterone does not exhibit the antioxidant activity as estrogen, progesterone displays a membrane stabilizing effect that also serves to reduce damage caused by lipid peroxidation (Roof and Hall, 2000). In addition, both estrogen and progesterone exert antioxidant actions indirectly through their ability to stimulate the expression of mitochondrially localized gene products. These proteins include SOD2 and Bcl-2 that in turn display direct and indirect antioxidant activities, respectively (Kowaltowski *et al*, 2004; Tripanichkul *et al*, 2007; Matsuoka *et al*, 2010).

A number of neuroprotective antioxidants have been chemically designed specifically to target mitochondria. This approach generally involves conjugation between the antioxidant and a lipophilic cation, e.g., tetraphenylphosphonium cation. The

lipophilicity promotes diffusion across the blood brain barrier and neural cell membranes while the positive charge promotes accumulation at the inner membrane or in the mitochondrial matrix, due to the very high, 180 mV negative-inside mitochondrial membrane potential. Antioxidants as diverse as ubiquinone, lipoic acid, vitamin E, and nitron spin-traps have been targeted to mitochondria in this manner (Murphy and Smith, 2007) and have shown neuroprotection in many models including those for neurodegenerative diseases and alcohol neurotoxicity (Siler-Marsiglio *et al*, 2005). Both mitochondrially targeted ubiquinone and a nitron spin-trap have shown efficacy in a neonatal rat cerebral hypoxic ischemia model (Hobbs *et al*, 2008), which is particularly significant since endogenous antioxidant levels in the neonatal brain and neonatal brain mitochondria are considerably lower than those present in adult brain (Bayir *et al*, 2006).

Increasing mitochondrial antioxidant activities by elevating mitochondrial reducing power is considered as an important mechanism of neuroprotection by several naturally occurring compounds, including ketone bodies, pyruvate, and acyl-carnitines (Ryu *et al*, 2004; Zanelli *et al*, 2005; Gasior *et al*, 2006; Zhao *et al*, 2006b; Jarrett *et al*, 2008; Rosca *et al*, 2009; Alves *et al*, 2009; Jones *et al*, 2010; Zhang *et al*, 2010). This mechanism of action might be particularly important during reperfusion after cerebral ischemia, when the mitochondrial redox state is hyperoxidized, ROS production is elevated, and there are increased markers of oxidative molecular modification (Perez-Pinzon *et al*, 1999; Fiskum *et al*, 2004). There is evidence for mitochondrial metabolism of both ketone bodies, e.g., β -hydroxybutyrate, and acetyl-L-carnitine after acute brain injury, which is associated with a reduction in oxidative stress and neuroprotection (Rosenthal *et al*, 1992; Liu *et al*, 1993; Prins *et al*, 2005; Scafidi *et al*, 2010, 2011). While there is less evidence that neuroprotection by exogenous pyruvate is a consequence of its oxidative metabolism, support for this mechanism comes from recent measurements of brain slice O_2 consumption indicating that exogenous pyruvate significantly elevates endogenous respiration (Schuh *et al*, 2011). Other experiments indicate that each of these three neuroprotectants exhibit alternative mechanisms of neuroprotection, including direct scavenging of free radicals (Packer *et al*, 1991; Varma *et al*, 1998; Haces *et al*, 2008). Moreover, one study indicates that ketone bodies inhibit mitochondrial ROS production in neurons after glutamate excitotoxicity by decreasing mitochondrial redox state (Maalouf *et al*, 2007). More research is clearly necessary to elucidate the mechanisms of neuroprotection by these relatively very safe, naturally occurring substances.

The levels of gene products that are responsible both for detoxification of reactive O_2 species and for the reducing power that drives their detoxification are controlled through transcriptional regulation

using antioxidant response elements (ARE) that interact with transcriptional activating factors such as Nrf2 (Thimmulappa *et al*, 2002; Figure 5). One mechanism by which the Nrf2/ARE pathway of antioxidant- and other cytoprotective-gene expression is activated is oxidation of critical cysteine sulfhydryl groups located on KEAP1 (Kelch-like ECH-associated protein 1), a cytoplasmic Nrf2 binding protein. On oxidation of KEAP1, Nrf2 is released from this protein, undergoes serine phosphorylation, and translocates to the nucleus where it binds to AREs (Jaiswal, 2004).

Sulforaphane, an isothiocyanate derived from a glucosinolate found in cruciferous vegetables, e.g., broccoli, forms mixed disulfide bonds with KEAP1, and is a well-studied pharmacologic activator of Nrf2-mediated gene expression (Zhang *et al*, 1992; Kensler *et al*, 2000). Sulforaphane shows neuroprotection in several rat models of acute brain injury, e.g., stroke (Zhao *et al*, 2006a) and head trauma (Zhao *et al*, 2005), in which evidence for mitochondrial PTP involvement exists (Kristian and Siesjo, 1998; Okonkwo and Povlishock, 1999). Rats fed a

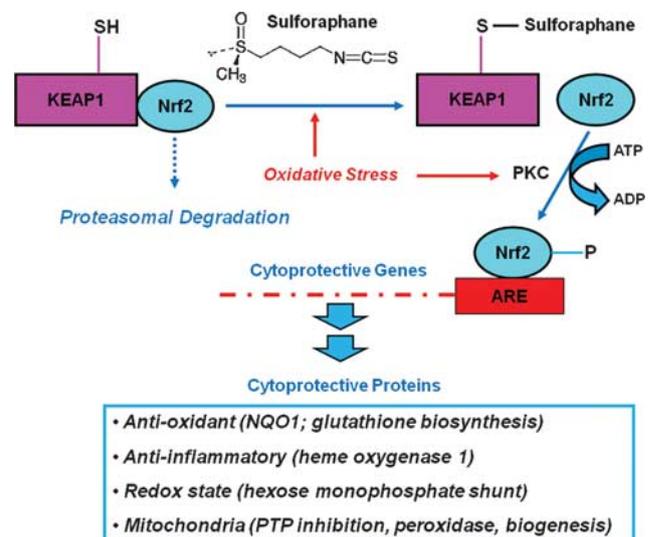


Figure 5 Neuroprotection by Nrf2-stimulated expression of cytoprotective genes. In unstressed cells, Nrf2 is bound to its inhibitory protein, Kelch-like ECH-associated protein 1 (KEAP 1), which targets it for proteasomal degradation. Oxidation of specific KEAP1 cysteine sulfhydryl groups in response to either oxidative stress or mixed dithiol reactions with compounds, e.g., sulforaphane, induces a conformational change in KEAP1, reducing its ability to bind Nrf2. Following serine phosphorylation of Nrf2 by kinases, e.g., protein kinase C (PKC) delta, Nrf2 can enter the nucleus where it binds to antioxidant response elements (ARE) in the promoter regions of many cytoprotective genes. Examples of gene products that contribute to neuroprotection by Nrf2 activation include those present in the cytosol, e.g., NADPH/CoQ oxidoreductase 1, and those present in the mitochondria, e.g., thioredoxin and NADP⁺-dependent malic enzyme. Changes in mitochondrial antioxidant-associated proteins appear responsible for inhibition of permeability transition pore (PTP) opening in brain and liver mitochondria from animals treated with Nrf2 activators, e.g., sulforaphane.

broccoli-enriched diet exhibit significant increases in aortic smooth muscle mitochondrial proteins that could influence PTP opening, including thioredoxin, thioredoxin reductase, glutathione reductase, glutathione, and catalase enzyme activities (Mukherjee *et al*, 2008). Mitochondria isolated from the livers of rats injected intraperitoneally 40 hours earlier with sulforaphane exhibit elevated immunoreactive levels of glutathione peroxidase 1, thioredoxin 2, and mitochondrial malic enzyme (Greco *et al*, 2011). These mitochondria are characterized by elevated peroxidase enzyme activity, increased glutathione, and resistance to NAD(P)H oxidation induced by exposure to peroxides. These investigators also found that both the liver and brain mitochondria isolated from sulforaphane-treated rats are resistant to PTP opening induced by exposure to Ca^{2+} plus peroxides (Greco and Fiskum, 2010a). Considering the importance of PTP opening in the pathophysiology of both acute central nervous system injury and possibly also in neurodegenerative diseases, the ability of sulforaphane and other Nrf2 activators to increase mitochondrial antioxidant proteins inhibits the opening of this mitochondrial death pore may represent a powerful new approach toward neuroprotection.

The Nrf2/ARE pathway is not the only approach toward cytoprotection through elevation of mitochondrial antioxidant-related proteins. For instance, pharmacologic activation of the STAT3 stimulates the expression of the mitochondrial SOD2 and limits ischemic neuronal death (Jung *et al*, 2009, 2010). Considering the fact that SOD2 immunoreactivity is not increased in brain mitochondria from rats treated with the Nrf2 activator sulforaphane, a combination of Nrf2 and STAT3 activators could provide mitochondria with a greater resistance to oxidative stress than following the administration of activator alone.

Summary and Conclusions

Mitochondria are in many ways like cells within cells, possessing their own genome, the ability to replicate, and molecular mechanisms for responding to changes in their environment. The mitochondrial presence of ~1,000 different proteins, two very different membranes, numerous transporters for ions, metabolites, and proteins provide numerous targets for both injury and cytoprotection. Like injured cells, damaged mitochondria can poison their immediate environment, ultimately leading to the death of the host cells in which they reside. There are many strategies for protecting cells such as neurons against pathologic mitochondrial injury. This review focused on three approaches: (1) Optimization of mitochondrial dynamics through mitophagic disposal of abnormal mitochondria and biogenesis of new, healthy mitochondria. (2) Utilization of ischemic-preconditioning mechanisms such as mitochondrial protein phosphorylation and sir-

tuin activation that improve mitochondrial bioenergetics. (3) Pharmacologic inhibition of mitochondrial oxidative stress by inhibition of mitochondrial superoxide production, by mitochondrially targeted antioxidants, and by stimulation of antioxidant gene expression that increases endogenous mitochondrial antioxidant defenses. Overlap between these three approaches clearly exists. Agents or conditions that activate all three pathways may have particularly strong potential for neuroprotection. The possibility also exists, however, that negative interactions could limit the effectiveness of combination therapies. For instance, Nrf2 activation results in a shift in cellular redox state to a more reduced level (del *et al*, 2008; Greco *et al*, 2011), which could inhibit the effectiveness of neuroprotective approaches mediated by sirtuins, that are activated by an oxidized shift in redox state. Furthermore, considering the finding that histone deacetylase inhibitors both activate Nrf2 and protects against ischemic brain injury (Kawai *et al*, 2011; Wang *et al*, 2011), activation of sirtuin histone deacetylase activity could potentially impair Nrf2-mediated cytoprotective gene expression. A myriad of other synergistic and antagonistic interactions could exist between agents or conditions that exert mitochondria-based neuroprotection through different molecular mechanisms. These interactions are likely to be dependent on dose, timing of administration, and the neuropathologic paradigms in which these approaches are tested.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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