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SILDENAFIL CAUSES A LARGE NEUROPROTECTION AGAINST MALONATE-INDUCED LESION OF THE RAT STRIATUM

Diploma thesis

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Malonate

It is clear that impairment of mitochondrial energy metabolism is the key pathogenic factor in a number of neurodegenerative disorders (Schon and Manfredi, 2003). Accordingly, toxins that affect mitochondria are being used as pharmacological tools to mimic several of these diseases. Among others, 3-NP, MPTP, rotenone and malonate are well-established mitochondrial complex inhibitors frequently used to investigate the key cellular pathways that provoke neurodegeneration in Parkinson's (PD) or Huntington's (HD) diseases (Browne and Beal, 2002), In addition, histological characterization of lesions produced by malonate has revealed striking similarities to "excitotoxic" lesions observed in some neurological diseases, such as focal ischemia and HD (Browne and Beal, 2002; Greene and Greenamyre,1996). The mechanisms that account for these neurotoxic effects remain to be fully elucidated (Ferger et al., 1999).

Malonate is a reversible inhibitor of mitochondrial succinate dehydrogenase (SDH) and consequently can induce mitochondrial dysfunction (see figure 1). In fact, malonate can trigger the generation of superoxide radicals, secondary excitotoxicity mediated by Ca^{2+} influx, and apoptosis (Dedeoglu et al. 2002). It has been reported that malonate is capable of inducing a caspase-dependent apoptotic cell death (Schulz et al., 1998). Mitochondria are being considered a main link between cellular stress signals activated during acute and chronic nerve cell injury and the execution of apoptotic and necrotic cell death (Jordan et al. 2004; Mattson and Kroemer, 2003). ATP depletion, pathophysiological increases in intracellular calcium (Ca^{2+}) and enhanced reactive oxygen species (ROS) production frequently occur during necrotic cell death and can trigger an increase in the permeability of the inner mitochondrial membrane. This process is believed to involve the formation of a multiprotein channel referred to as "mitochondrial permeability transitory pore" (Bernardi, 1999), which triggers the release of solutes up to 1500 Da from the mitochondrial matrix into the cytoplasm.

Apoptotic events in contrast can cause an increase in the permeability of the outer mitochondrial membrane (Green, 2006), but the whole problem is discussed later.

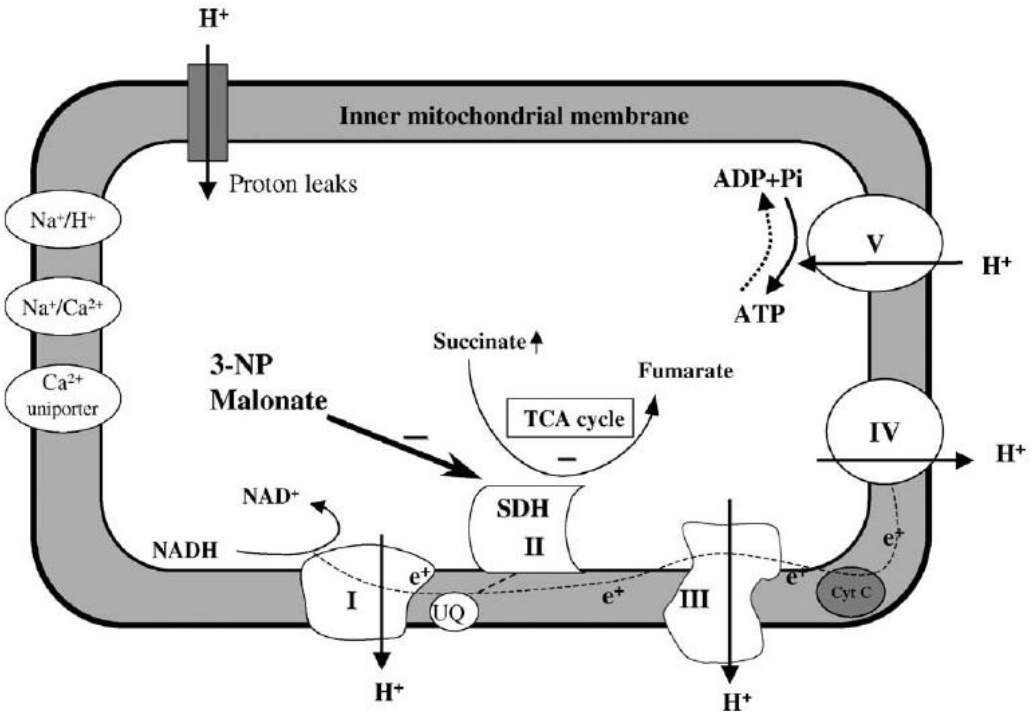

Fig.1. *The structure of mitochondrion showing the components of the respiratory chain and the site of 3-NP and malonate inhibition in respiratory chain and TCA cycle. In normal condition, complexes I, III, and IV function as proton pumps, and complex V (ATP synthase) is the dominant pathway for the reentry of protons into mitochondrial matrix, which maintains the mitochondrial membrane potential and leads to the synthesis of ATP (solid arrow).Under succinate dehydrogenase (SDH; complex II) inhibition or the application of protonophores (proton leaks), there is a collapse of mitochondrial membrane potential, which reverses the ATP synthase (dotted arrow) and leads to the hydrolysis of cytoplasmic ATP. The depletion of cytoplasmic ATP will further prevent the Na+ and Ca^{2+} extrusion from the neurons, and lead to a subsequent failure of glycolysis. Because SDH is also one of the components in TCA cycle, the inhibition of SDH will also result in the elevation of succinate as shown in the figure (Na^{+}/H^{+} exchanger; Na^{+}/Ca^{2+} exchanger; UQ, ubiquinone; Cyt C, cytochrome c). Adapted fom W.-T. Lee, C. Chang / Progress in Neurobiology 72 (2004) 87–110.*

Futhermore several earlier studies have demonstrated that the neurotoxicity associated with intrastriatal injection of malonate is mediated by the indirect activation of N-methyl-D-aspartate (NMDA) receptors (Greene et al.1995, Henshaw et al. 1994, Beal et al. 1993).

In any case various mechanisms underlying malonate-induced neurotoxicity in many scientific articles have been discussed but many questions still remain unclear.

Huntington's disease

Huntington‘s disease (HD), an autosomal dominant neurodegenerative disorder, is characterized clinically by progressive cognitive impairment, abnormalities of movement, and neuropsychiatric symptoms (Marshall and Shoulson, 1997). Selective neuronal loss, predominantly in the striatum and other basal ganglia structures (see figure 2), accounts for the majority of the clinical features of Huntington‘s disease (Vonsattel et al., 1985). Although the average age of onset is approximately 40 years (Adams et al., 1988, Farrer et al., 1985), the disease may begin any time from childhood to old age. The number of cytosine-adenosine-guanosine (CAG) repeats within the Huntington‘s disease gene accounts for much of this variation in age of onset.

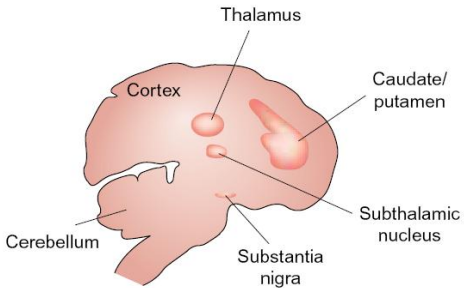

Fig.2. *Cartoon of a sagittal section of human brain showing major sites of neuronal loss in HD. Adapted from David C. Rubinsztein / TRENDS in Genetics Vol.18 No.4., 2002.*

The exact mechanisms underlying neuronal death in Huntington‘s disease are unknown. Over the past 10 years, the leading models of neurodegeneration in the disease have involved mitochondrial dysfunction and subsequent excitotoxic injury, oxidative stress, and apoptosis. Recent studies have lent support to these models, but additional theories involving abnormalities of protein metabolism and transcriptional dysregulation have emerged as well. As progress is made toward clarifying the pathophysiological mechanisms leading to Huntington‘s disease, and new therapies are proposed, investigators have begun to develop improved outcome measures for potential use in future clinical trials aimed at slowing disease progression. Recent advances in understanding the pathophysiology of Huntingdon‘s disease

have focused on the trinucleotide expansion, transcriptional regulation, protein aggregation, mitochondrial function, excitotoxicity, and apoptosis.

Trinucleotide Expansion

A CAG (cytosine-adenosine-guanine) repeat expansion greater than 38 within exon of the Huntingtin gene results in the development of the disease (Duyao et al.,1993). The gene is called IT15 and was identified as the causative gene for HD in 1993 (Huntington's Disease Collaborative Research Group, 1993).

CAG codes for glutamine and the Huntington's disease mutation results in an expanded polyglutamine tract at the amino terminus of the Huntingtin protein (Htt). The underlying cause for the unstable expansion of the CAG repeat sequence remains unknown, but the mechanism that produces the expansion in the Huntington's disease gene may also affect other genes in patients with the disease (Keckarevic et al., 2000), play an important role in juvenile onset of HD (Macdonald et al., 1993) or aging (Mangiarini et al., 2001; McMurray et al., 2001).

Transcriptional Regulation

The expanded trinucleotide repeat in the Huntington's disease gene may lead to abnormalities of genetic transcription (Cha, 2000). Many transcription factors contain polyglutamine stretches, so mutant Htt may gain an abnormal transcriptional function, either to enhance transcription of some genes, or perhaps by binding to existing transcription factors, to repress transcription (Boutell et al., 1999). In fact, impaired transcription of the gene for brain derived neurotrophic factor (BDNF), which is essential for the maintenance and differentiation of neurons in the brain, has been described in Huntington's disease (Zuccato et al., 2001). Other genes for which transcription appears to be altered include genes for neurotransmitter receptors, intracellular signaling proteins, and proteins involved in calcium homeostasis (Cha, 2000). These observations suggest that abnormalities of transcription may underlie the pathogenesis of the disease and raise the very exciting possibility of the treatment of Huntington's disease.

Protein Aggregation

Intranuclear and cytoplasmic aggregates of mutant Htt have been identified in both human Huntington's disease brains and in transgenic animal models of the disease (Davies et al., 1997; Difiglia et al., 1997), but the role of these inclusions in the pathogenesis remains

unknown. Some studies have found that neurodegeneration precedes the development of aggregates (Guidetti et al., 2001), perhaps suggesting that aggregate formation is not pathogenic. Many proteins have been reported to bind to both normal and mutant Htt (Burke et al., 1996; Kalchman et al., 1996), and in many cases, the extent of binding is related to the number of CAG repeats. Which, if any, of these interactions contributes to pathogenesis remains uncertain.

Normal Htt has been shown to protect striatal cells from serum deprivation and from exposure to the mitochondrial toxins 3-nitropropionic acid or malonate (Beal et al., 2000). The mechanism of this neuroprotective property of Htt appears to be via an anti-apoptotic effect mediated through the inhibition of procaspase-9 (Rigamonti et al., 2001; Rigamonti et al., 2000.). Mutant Htt lacks this neuroprotective quality, and by binding normal Htt may render striatal neurons susceptible to injury. These observations raise the possibility that Huntington‘s disease may in part be caused by a loss of the normal function of Htt, as opposed to the long-held belief that the Huntington‘s disease mutation results in a toxic gain of function.

Mitochondrial Function

A defect in mitochondrial energy production leading to increased susceptibility to excitotoxic injury has long been implicated in the pathogenesis of Huntington‘s disease (Beal et al., 1992). Evidence for impaired mitochondrial function includes (1) mitochondrial electron transport inhibitors such as 3-nitropropionic acid and malonate produce brain lesions in animals that mimic the histopathological changes seen in Huntington‘s disease (Beal et al.,1992); (2) these lesions are accompanied by behavioral and motor changes similar to those seen in Huntington‘s disease patients (Kodsi et al., 1997; Borlongan et al.,1995); (3) decreased activity of complexes II and III of the electron transport chain in the caudate in post-mortem diseased brain (Kodsi et al., 1997); (4) decreased complex I activity in platelets and muscle of patients, the severity of which may correlate with CAG repeat length (Arenas et al., 1998; Parker et al., 1990); and (5) the identification of increased lactate levels in the brain of patients using magnetic resonance spectroscopy, suggesting an increase in anaerobic energy metabolism (Harms et al., 1997; Jenkins et al., 1993). Abnormalities of mitochondrial energy production may render striatal neurons susceptible to excitotoxic injury.

Excitotoxicity

Evidence that glutamate mediated excitotoxicity, via the N-methyl-D-aspartate (NMDA) receptor, may be involved in the neurodegenerative process in Huntington‘s disease includes the following: (1) NMDA receptor bearing neurons appear to be relatively selectively lost in the disease, (2) Intrastriatal injections of NMDA receptor agonists produce lesions that mimic the lesions seen in Huntington‘s disease, and these lesions can be attenuated by NMDA receptor antagonists (Beal et al., 1993). (3) Increased glutamate transporter messenger RNA expression is observed in astrocytes of post-mortem brain, perhaps representing a compensatory mechanism to limit glutamate excitotoxicity (Arzberger et al., 1997). More recently, mutant Htt has been found to enhance excitotoxic cell death (Zeron et al., 2001).

Apoptosis Cascades

Programmed cell death or apoptosis has also been implicated in the pathogenesis of Huntington‘s disease. The cascade of reactions that are part of apoptosis are largely mediated by caspase activity (Earnshaw et al.,2000). Caspases play an intimate role in apoptosis that induces cell death (Hickey et al.,2003; Thornberry et al.,1997) by indirectly or directly initiating apoptosis (Friedlander and Robert, 2003).

Two major pathways exist by which initiator caspases lead to cell death - one is extrinsic, the other intrinsic. Because of our scientific interest in apoptotic cascades we discuss this problem more deeply below.

Apoptosis

Intrinsic and Extrinsic Apoptotic Pathways

In multicellular organisms, elimination of excess and damaged cells is highly regulated and occurs through a genetically controlled pathway known as programmed cell death (PCD) or apoptosis. PCD is evolutionary conserved and its execution relies on activation of caspases, a family of aspartyl-directed cysteine proteases. Activation of executioner caspases (caspase-3, -7, and -6) is strictly regulated and initiated through cleavage by upstream initiator caspases (caspase -8, -9, -10, and -12) (Adams, 2003; Reed, 2004). Two major pathways of caspase activation have been discovered in mammalian cells: the intrinsic or mitochondrial pathway, and the extrinsic or death receptor pathway (see figure 3).

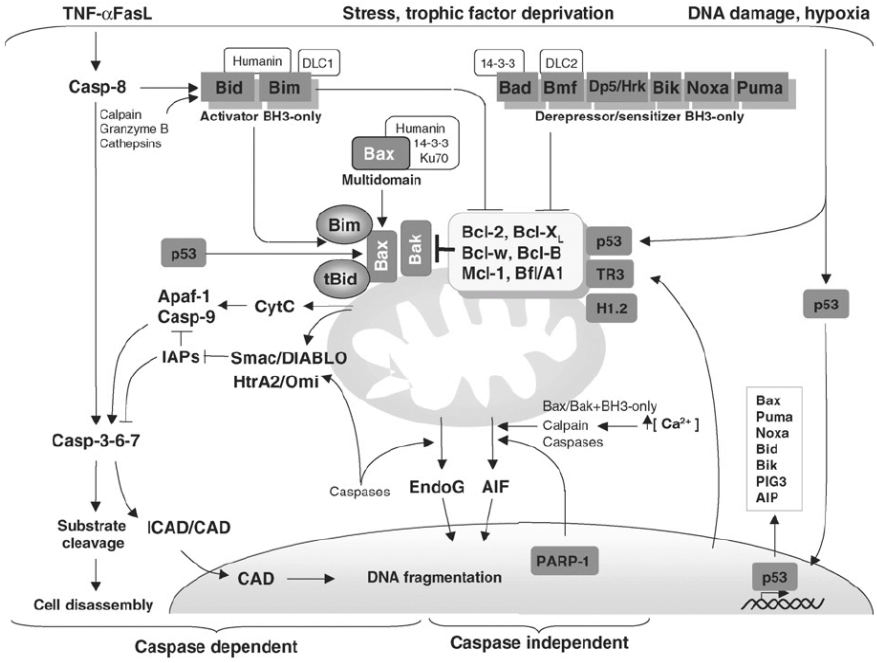

Fig.3. *Schematic representation of the intrinsic (mitochondrial) and extrinsic (death receptor) pathways of apoptosis, including cytochrome c and Bcl-2 family proteins. Adapted from L. Soane et al., Mitochondrial mechanisms of oxidative stress and apoptosis (2007).*

The discovery that cytochrome c (CytC) release from mitochondria is required for apoptosis (Liu et al., 1996) revealed a new and unexpected function of mitochondria. In addition to its bioenergetic function, mitochondria are also the source of potent apoptotic proteins. The key event that links mitochondria to the activation of caspases is the permeabilization of the outer mitochondrial membrane (OMM) resulting in the release of CytC and other apoptogenic proteins (apoptosis-inducing factor (AIF), Smac/DIABLO, endonuclease G (EndoG), and HtrA2/Omi) into the cytosol (Lindholm et al., 2004). In the presence of ATP, CytC binds to Apaf-1 (apoptotic protease-activating factor-1 and triggers activation of the initiator procaspase-9 within the apoptosome (Zou et al., 1997). Active caspase-9 then activates effector caspase-3 and -7 that in turn cleave numerous substrates responsible for DNA fragmentation and cell disassembly. Activation of caspase-3 and -9 is endogenously controlled by the inhibitor of apoptosis (IAP) family proteins. The IAP inhibitory step is mitigated through the release of the serine protease HtrA2/Omi and Smac/DIABLO. AIF and EndoG are also released from mitochondria, and can mediate a caspase-independent mitochondrial death pathway by promoting DNA fragmentation. The complex role of mitochondria in apoptosis is highlighted by the dual role, pro-death or pro-survival, of some apoptogenic proteins, e.g., AIF and HtrA2/Omi, that induce cell death when released from mitochondria, but promote neuronal survival and resistance to oxidative stress at their mitochondrial location (Lindholm et al., 2004). PCD occurs as a natural process in the nervous system during development (Lossi and Merighi, 2003) and a role for PCD in physiological aging is also suggested (Taglialatela et al., 1996). Developmental death in various regions of the brain (e.g., cerebellum, retina) occurs at early developmental stages for neural precursor cells (NPC), and later during the period of synaptogenesis, in postmitotic neurons establishing proper connections. Much of this developmental death appears to be apoptotic (Lossi and Merighi, 2003). Evidence of apoptotic death through the intrinsic pathway is found in acute (e.g., trauma, stroke) and chronic neurodegenerative disorders (e.g. Alzheimer disease (AD)) (Waldmeier, 2003).

Apoptotic neuronal death can also proceed through the extrinsic pathway or death receptor pathway, initiated by the binding of structurally related extracellular death ligands (e.g., tumor necrosis factor (TNF)-α, FasL/CD95L) to the TNF receptor/death receptor (DR) family (Khosravi-Far and Esposti, 2004). Ligation of DR results in activation of initiator caspase-8 that can efficiently activate caspase-3 in some cells types (type I). In other cell types (type II), the mitochondrial pathway is recruited through caspase-8-mediated cleavage

of proapoptotic Bid that translocates to mitochondria and activates Bax and Bak. This pathway can be induced in some neurons in response to trophic factor deprivation, which in many acute (e.g., ischemia, trauma) and chronic CNS disorders (e.g., AD, HD and PD) occurs.

Bax, a Member of Bcl-2 Family Proteins

Most important regulators of the intrinsic pathway, Bcl-2 (B cell lymphoma) family proteins, are expressed in both the embryonic and adult CNS, and control neuronal death during development and in pathologic conditions. Bcl-2 family consists of pro- and antiapoptotic proteins, characterized by the presence of at least one of the four Bcl-2 homology (BH) domains. Proapoptotic Bcl-2 proteins are subdivided into multidomain (BH1–3) proteins (Bax, Bak, and Bok/Mtd) and BH3-only proteins (Bid, Bim, Bad, Bmf, Dp5/Hrk, Puma, Noxa, Bik, Bnip3) as shown in figure 4.

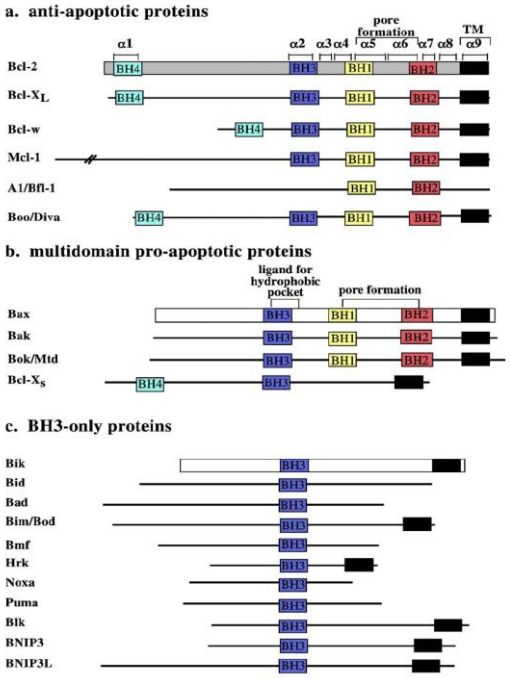

Fig.4. *Representation of all the known mammalian Bcl-2 family members. Bcl-2 homology regions 1–4 (BH1–4) are indicated. TM indicates a putative transmembrane region that mediates localization to intracellular membranes. α: Hα. The Hα5–Hα6 overlapping region containing the BH1 domain corresponds to the pore-forming region based on structural homology with bacterial toxins. The BH3 domain in the pro-apoptotic members is a ligand for the hydrophobic pocket formed by the BH1–BH3 domains of the anti-apoptotic members. Adapted from Er et al., Biochimica et Biophysica Acta 1757 (2006), 1301–1311.*

Bcl-2 family proteins regulate cell death pathways by controlling release of proapoptotic proteins from mitochondrial intermembrane space (IMS) through regulation of the OMM permeability. A rise in OMM permeability is triggered by multidomain Bax/Bak in cooperation with BH3-only proteins. In contrast, OMM permeabilization is inhibited by antiapoptotic members (e.g., Bcl-2, Bcl-XL). While the exact biochemical activities are not completely elucidated, the BH3-domain-dependent heterodimerization is critical for proapoptotic activity. Proteins sharing this structural fold can insert into lipid membranes and form ion channels (Schendel et al., 1998). While the role of ion channel formation is unclear, Bax/Bak can also form large oligomers, which facilitate the release of CytC through the OMM (Antonsson et al., 2000).

Prior to a death stimulus, monomeric Bax can reside in the cytosol in an inactive conformation, bound to different proteins (Nomura et al., 2003; Guo et al., 2003) or it can be loosely attached to mitochondria. Bak normally resides in mitochondria (Cheng et al., 2003). Allosteric activation of Bax results from a conformational change that exposes its N terminus. This promotes its translocation to the mitochondria, stable OMM insertion, oligomerization, and subsequent release of apoptogenic proteins from the IMS (Desagher et al., 1999). The exact mechanism of Bax/Bak-mediated OMM permeabilization is still a matter of debate.

Reconstitution experiments demonstrate that Bax and a BH3-only protein (e.g., tBid) are the minimal components required for CytC release (Kuwana et al., 2002). Activated tetrameric Bax can form pores sufficiently large to allow passage of CytC (Saito et al., 2000). tBid-induced oligomerization of Bax is required for the release of liposome-entrapped molecules, and Bax oligomers are detected in apoptotic cells (Antonsson et al., 2000). Cooperation with mitochondrial membrane lipids, particularly cardiolipin (CL), appears to play a role in this process (Kuwana et al., 2002). CL, an anionic phospholipid exclusively present in mitochondria of eukaryotic cells, might confer specificity for targeting of mitochondria by tBid, and is required for Bax-mediated pore formation. CL is localized primarily in the inner membrane and is required for CytC binding. Therefore, its role might be to regulate the extent of CytC release after OMM permeabilization (Iverson and Orrenius, 2004). Bax/BH3 domain peptide-mediated CytC release involves a selective permeabilization of the OMM without loss of the inner mitochondrial membrane integrity loss (Polster et al., 2001).

Studies using genetically modified animals have uncovered essential roles for proapoptotic Bcl-2 family proteins in naturally occurring developmental death and neuronal death

associated with neurodegeneration (Akhtar et al., 2004; Lindsten et al., 2005). Targeted deletion of Bax or Bak does not result in major developmental defects, however, a reduction in apoptosis of neurons in the brainstem, hippocampus, cerebellum, dorsal root ganglia, and spinal cord is observed in Bax-deficient mice. Therefore, these proteins are apparently involved in pathologic apoptosis and nowadays serve as the very interesting topic for reseach of neurodegenerative diseases.

p38 Mitogen-Activated Protein Kinase

Cells are always required to integrate external stress signals and thus decide cell fates to die or survive on an ongoing basis. These fate decisions are made by a wide range of signaling pathways that are controlled by kinases. Developmental programs and environmental agents trigger distinct and evolutionarily conserved kinases (Caffrey et al., 1999; Widmann et al., 1999) that relay signals mediating proliferation, survival, death, or cell cycle arrest. The mitogen-activated protein kinases (MAPKs) are the family of kinases that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli, including stress (Johnson and Lapadat, 2002; Werlen et al., 2003). Conventional MAPKs consist of three family members: the extracellular signal-regulated kinase (ERK), the c-Jun NH2-terminal kinase (JNK) and the p38-MAPK. Each family member has its own subfamilies: ERKs (ERK1 and ERK2), JNKs (JNK1, JNK2, and JNK3) and p38-MAPKs (p38-MAPKa, p38-MAPKb, p38-MAPKc, and p38-MAPKd) (Chang and Karin, 2001; Johnson and Lapadat, 2002). The p38 kinases are highly conserved during evolution, the human p38 kinases are 99% identical to mouse. MAPKs are serine/threonine kinases that are widely expressed in many tissues including brain and are activated by dual phosphorylation on Threonin 180 and Tyrosin 182 (Raingeaud et al., 1995). This phosphorylation is mediated by a signalling cascade (Whitmarsh and Davis, 1996) containing the MAP kinase kinases MKK3 (Derijard et al., 1995) and MKK6 (Raingeaud et al., 1996).

Such phosphorylation events can either positively or negatively influence substrate regulation, and thus the entire signaling cascade activity. Thus, the MAPK signaling pathways modulate gene expression, mitosis, proliferation, motility, metabolism, and programmed cell death ‘apoptosis’ (Johnson and Lapadat, 2002; Werlen et al., 2003).

In fact there is a significant body of evidence suggesting that p38 MAPK activation plays an important role during excitototoxic and neurodegenerative processes. The p38 MAP kinase participates in several apoptosis pathways, mediating Bax activation and translocation

(Ghatan et al., 2000). But the meaning of the information on p38 kinase that has been mainly provided by in vitro experiments and has to be dealt with care.

Oxidative Stress

Contribution of Oxidative Stress to Neurodegeneration

Several lines of evidence indicate that oxidative stress is a primary mechanism of neuronal cell death associated with a wide range of neurologic disorders. The extent of delayed neuronal death correlates well with prelethal markers of oxidative molecular alterations to lipids, proteins, and nucleic acids in both animal models and patients (Cristofori et al., 2005; Musiek et al., 2005). Moreover, these oxidative modifications are also observed at the mitochondrial level (Hsu et al., 2005; Naoi et al., 2005). Neuroprotection occurs following the use of antioxidants and inhibitors of free radical-producing enzymes, some of which target mitochondria (Beal, 2004; Park et al., 2006). In addition, genetic animal models where antioxidant enzymes, including the mitochondrial enzymes manganese superoxide dismutase (SOD2) and glutathione peroxidase (GPx), are either over- or underexpressed display increased and decreased resistance, respectively, to acute and chronic neurodegeneration (McLean et al., 2005; Moskovitz, 2005).

Mitochondria as Sources of Reactive Oxygen Species

Superoxide is a normal byproduct of mitochondrial electron transport respiration and is responsible for 0.2–2.0% of mitochondrial and therefore cellular O_2 consumption (Barja, 1999; St Pierre et al., 2002). Due to its extremely short half-life, superoxide normally reacts with itself to form H_2O_2, aided by superoxide dismutase 1 (SOD1) and SOD2 in the cytosol and mitochondria, respectively. The metabolism of H_2O_2 to H_2O via GPx and other peroxidases can result in oxidative stress if this causes a net oxidized shift in the redox state of glutathione and pyridine nucleotides (NAD(H) and NADP(H)). As NADPH is the direct reductant responsible for maintaining reduced glutathione (GSH) via glutathione reductase, the reduction of $NADP^+$ by mitochondrial isocitrate dehydrogenase, malic enzyme, and nicotinamide nucleotide transhydrogenase reactions is critical for detoxification of intramitochondrial peroxides (Vogel et al., 1999). GSH can also be irreversibly lost without an increase in GSSG in brain mitochondria due to formation of protein-SS-glutathione mixed disulfides (Ravindranath and Reed, 1990).

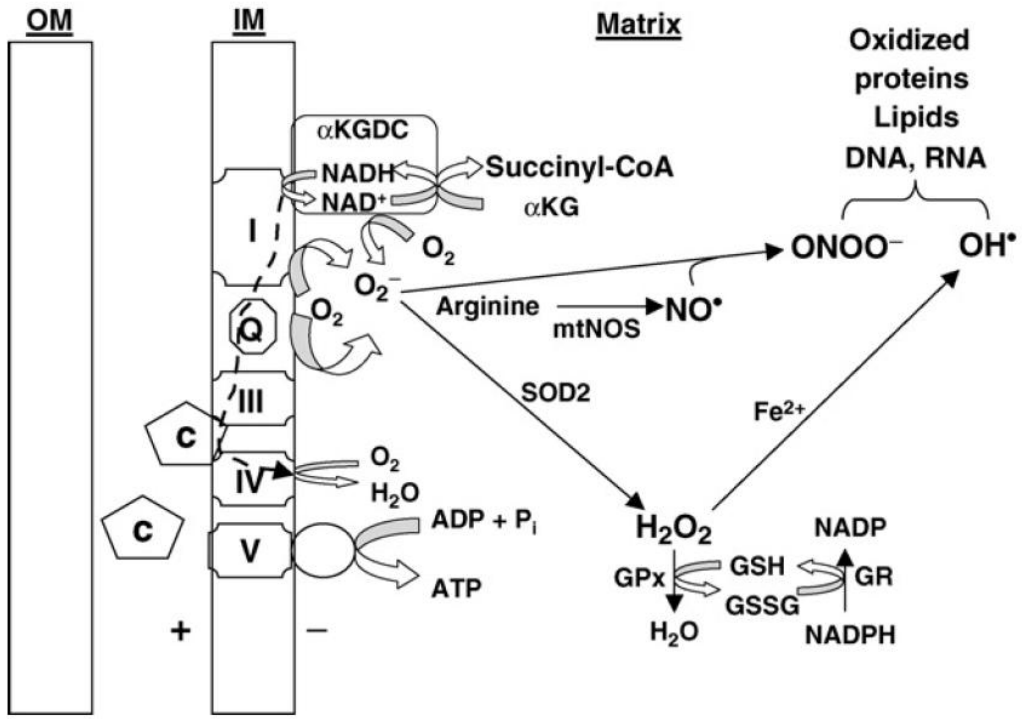

Fig.5. *Mitochondrial generation and metabolism of reactive oxygen species (ROS). Normally, around 99% of the O_2 consumed by mitochondria occurs at the ETC complex IV (cytochrome oxidase), driven by electrons ultimately donated to the ETC by dehydrogenases, e.g., α-ketoglutarate dehydrogenase complex (αKGDC) and succinate dehydrogenase (complex II, not shown). Some O_2 is also reduced by a single electron reaction, forming superoxide (O_2^-), catalyzed by reactions at ETC complexes I–III and by one or more dehydrogenases, e.g., αKGDC. Superoxide is dismutated to H_2O_2 both spontaneously and enzymatically via the mitochondrial manganese superoxide dismutase (SOD2). H_2O_2 and other peroxides are further metabolized to H_2O or alcohol derivatives by one or more glutathione peroxidases (GPx). The electrons necessary for this reduction reaction come from reduced glutathione (GSH), which in turn is kept in its reduced state by glutathione reductase (GR). Excessive superoxide production occurs when the flow of electrons through the ETC is impaired by altered activities of any of the complexes due to genetic alterations, ETC inhibitory toxins (e.g., rotenone), or protein modifications (e.g., due to oxidative stress). Release of cytochrome c through the outer membrane also inhibits the ETC and promotes superoxide formation. Consequently, excessive H_2O_2 production can deplete mitochondria of reduced glutathione (GSH), resulting in the accumulation of lipid peroxides and oxidized proteins. In the presence of reduced iron (Fe^{2+}), the reduction of H_2O_2 leads to the formation of the highly reactive hydroxyl radical (OH^-). Nitric oxide (NO), generated either extramitochondrially or via the mitochondrial nitric oxide synthase (mtNOS), can react with superoxide, forming peroxynitrite ($ONOO^-$). Peroxynitrite and hydroxyl radical are capable of oxidizing virtually all cellular molecules, including many present at the mitochondrion that are essential for cell viability. Adapted from L. Soaneet al., Mitochondrial Mechanisms of Oxidative Stress and Apoptosis (2007).*

The chemical mechanisms responsible for mitochondrial superoxide formation are not well understood. While much evidence points to the single electron reduction of O_2 by semiubiquinone during its two-step oxidation at complex III and to reactions at one or more redox sites within complex I (Lambert and Brand, 2004; Andreyev et al., 2005), recent evidence also strongly suggests that mitochondrial matrix dehydrogenases, particularly 2-oxoglutarate dehydrogenase, contribute significantly to mitochondrial superoxide production (Starkov et al., 2004; Tretter and Adam-Vizi, 2004).

The physiological and pathological factors that regulate mitochondrial superoxide production are those that influence mitochondrial bioenergetics, and therefore the thermodynamic driving force behind superoxide production. In general, conditions that cause a net reduced shift in the redox state of the sites of superoxide formation result in increased production, and those that produce an oxidized shift in redox state, result in decreased production. The redox state is very sensitive to the rate of respiration; therefore, the rate of ROS production during maximal oxidative phosphorylation conditions (state 3 respiration) in the presence of NADH-linked oxidizable substrates, e.g., pyruvate or glutamate, is only 30% as fast as the rate observed at rest (state 4 respiration). At rest the redox state of all electron transport chain (ETC) and of matrix dehydrogenase redox centers is relatively reduced (Starkov and Fiskum, 2003). The link between oxidative phosphorylation and redox state is the generation of an electrochemical gradient of protons ($\Delta\mu_{H+}$) across the mitochondrial inner membrane by respiration-dependent proton efflux. Therefore, conditions, in addition to ATP synthase activation, that reduce $\Delta\mu_{H+}$ also lower the rate of superoxide generation. As the vast majority of the energy present in $\Delta\mu_{H+}$ is in the form of the membrane potential ($\Delta\Psi$), compared to the small pH gradient, activities or chemicals that drop $\Delta\Psi$ without impairing the flow of electrons through the ETC also lower the rate of superoxide generation. The concept of reducing mitochondrial ROS production by mild respiratory uncoupling has been used to explain the neuroprotection afforded in vitro and in vivo by the administration of uncoupling agents (e.g. lipid soluble weak acids) (Maragos et al., 2003; Jin et al., 2004).

The same rationale has been applied to the finding that increased expression of mitochondrial uncoupling proteins (UCPs) provides neuroprotection (Andrews et al., 2005; Conti et al., 2005). While the activation of these UCPs by markers of cellular stress, including free fatty acids and ROS, is well documented (Echtay et al., 2002), the actual participation of UCPs in mitochondrial uncoupling and decreased ROS production in cells or tissues is still controversial (Nicholls, 2001).

Another potential mechanism for decreasing is activation of the mitochondrial ATP-sensitive potassium channel, which has been reported to be highly expressed in brain mitochondria (Bajgar et al., 2001). Nevertheless, neuroprotection is afforded by pretreatment of neurons with pharmacologic activators of the mitochondrial [$\Delta\Psi$] ATP-sensitive channel blocker, diazoxide (Mattson and Kroemer, 2003; Teshima et al., 2003). Despite the fact that mild uncoupling (i.e., reduction in $\Delta\Psi$) normally reduces mitochondrial ROS formation, numerous reports suggest that the opening of this channel somehow increases mitochondrial ROS production (Busija et al., 2005; Liang et al., 2005). Activation of mitochondrial [$\Delta\Psi$] ATP channels may represent one of the primary mechanisms for ischemic preconditioning stress involving the generation of ROS as a downstream effect (Oldenburg et al., 2003). While mitochondrial $\Delta\Psi$ and redox state are normally controlled by metabolic demand, i.e., ATP synthase activity (Dimroth et al., 2003), a number of pathological conditions can override this control. For instance, inhibition of electron flow at any redox site distal to sites of superoxide formation will cause a reduced shift in the redox state of those sites, thereby promoting superoxide formation. Examples of ETC inhibitors that produce these effects are those that target either complex IV (cyanide, sodium azide, and NO), complex III (antimycin A), complex II (malonate and 3-nitropropionate), or complex I (rotenone and paraquat).

A controversial topic in this field is the involvement of mitochondrial Ca^{2+} in ROS production. Respiration-dependent mitochondrial Ca^{2+} uptake occurs via electrophoretic uniport, and Ca^{2+} release is mediated by both $Ca^{2+}/2H^{+}$ and $Ca^{2+}/2Na^{+}$antiports (Gunter et al., 2004). They importantly regulate intramitochondrial Ca^{2+} in a range that mediates key metabolic enzymes, e.g., pyruvate and 2-oxoglutarate dehydrogenases, thereby modulating oxidative phosphorylation. The fact that Ca^{2+} activates these dehydrogenases, resulting in a net reduced shift in mitochondrial redox state, may suggest that a physiological increase in Ca^{2+} could also increase mitochondrial superoxide formation, although this has yet to be demonstrated. A supraphysiologic increase in intramitochondrial Ca^{2+} has been proposed to account for elevated mitochondrial ROS production in a number of cell death paradigms, including neuronal excitotoxicity (Castilho et al., 1999) and neuronal apoptosis (Mattson, 1998). One possible mechanism responsible for Ca^{2+}-induced mitochondrial ROS production is the membrane permeability transition (MPT). The MPT is induced specifically at the inner membrane by excessive mitochondrial Ca^{2+} accumulation and is promoted by an oxidized mitochondrial redox state. When MPT is fully activated, solutes up to approximately 1.5 kDa

equilibrate across the membrane resulting in loss of mitochondrial metabolites (e.g., NAD(P)H, glutathione), and net uptake of osmolytes like K^+, resulting in swelling of the matrix, and typically, rupture of the outer membrane. Outer membrane disruption results in release of soluble intermembrane proteins, e.g., adenylate kinase or CytC (Crouser et al., 2003). Treatment with calcium chelators such as BAPTA can reduce mitochondrial ROS production and promote neuroprotection (Keller et al., 1998).

Loss of CytC can certainly stimulate mitochondrial generation of ROS by causing a reduced shift in mitochondrial redox sites associated with superoxide production (Kushnareva et al., 2002; Starkov et al., 2002). However, the MPT also causes a drop in $\Delta\Psi$ and a loss of mitochondrial pyridine nucleotides, both of which depress mitochondrial generation of ROS (Costantini et al., 1996). However, recent work from Batandier et al. suggests that even if mitochondrial NAD(H) were released into the cytosol in response to the MPT, their residual concentration in the mitochondrial matrix in equilibrium with the cytosolic pool could be sufficient to support substantial ROS production (Batandier et al., 2004). As the MPT also results in the release of mitochondrial matrix glutathione, this loss and impairment of the GPx/reductase system could explain a net increase in ROS and associated markers of oxidative stress during excitotoxicity and acute brain injury (Anderson and Sims, 2002). An attractive alternative explanation for Ca^{2+}-induced net ROS production is its potent direct inhibition of both mitochondrial GPx and reductase enzyme activities (Zoccarato et al., 2004). There are many other hypothesis refering to ROS generetion, its negative effects and possible neuroprotective effects against oxidative stress, but the amount of publications and scientific works is beyond the scope of this work.

Mitochondrial Antioxidant Systems

The pathologically important net production of ROS and RNS is the difference between their generation and detoxification. Mitochondria possesses many nonenzymatic and enzymatic antioxidant defenses and other enzyme activities that are protective against a variety of ROS and RNS and the products of their reactions with proteins, lipids, and nucleic acids.

Mitochondria possess a very active and highly inducible manganese SOD, also known as SOD2 (Macmillan-Crow and Cruthirds, 2001). This enzyme is found exclusively in the mitochondrial matrix (see figure 5) and is critical for cell viability. Overexpression of SOD2 leads to neuroprotection from excitotoxic- and NO-mediated damage (Gonzalez-Zulueta et

al., 1998). In neural cell lines, overexpression of SOD2 reduces peroxynitrite ($ONOO^-$) formed by NO-generating agents and protects against protein nitration, mitochondrial membrane depolarization, and apoptosis (Keller et al., 1998). A number of nonenzymatic superoxide scavenging molecules are present in mitochondria, although the degree to which they contribute to superoxide scavenging is unknown. These molecules include dihydrolipoic acid, ubiquinone, α-tocopherol, pyruvate, ascorbate, CytC, and glutathione.

Removal of H_2O_2 is important to avoid production of the hydroxyl radicals. The primary intramitochondrial system, the activities of which are responsible for the reduction of H_2O_2 and lipid peroxides (to water and alcohols accordingly), constitutes GPx and glutathione reductase (GR) (see figure 5). The normal concentration of intramitochondrial glutathione is high (~10 mM), suggesting it may also act as a direct antioxidant. Depletion of either the cytoplasmic or the mitochondrial glutathione pools can have significant impact on neuronal viability (Heales and Bolanos, 2002; Gegg et al., 2003). For example, mitochondrial glutathione pool depletion in astrocytes (that are exposed to NO donors) results in increased cell death compared with astrocytes with an intact pool of mitochondrial glutathione (Muyderman et al., 2004; Sims et al., 2004). GPx1 is the primary GPx isoform present in mitochondria (Esworthy et al., 1997). Overexpression of GPx1 inhibits CytC release, caspase activation, and neuronal death in experimental stroke (Hoehn et al., 2003). In addition to GPx1, mitochondria possess activity of hydroperoxide GPx (GPx4). Overexpression of this enzyme protects against cell death caused by the mitochondrial poisons cyanide and rotenone (Arai et al., 1999), and against apoptosis induced by the glycolytic inhibitor 2-deoxyglucose (Nakagawa, 2004). The activity of NADPH-dependent mitochondrial GR is necessary for reducing the oxidized glutathione generated by GPx reactions (see figure 5). This enzyme, as noted earlier, is highly sensitive to inhibition by elevated intramitochondrial Ca^{2+} (Zoccarato et al., 2004). Exposure of neural cells to an inhibitor of GR greatly increases their sensitivity to death caused by the stimulation of endogenous H_2O_2 production (Buckman et al., 1993).

Although not as well characterized as the GPx/reductase system, the mitochondrial thioredoxin/thioredoxin reductase and glutaredoxin/glutaredoxin reductase systems are also likely important for neuroprotection (Tanaka et al., 2000). These enzyme systems reduce protein disulfides and mixed disulfides, respectively. Finally, other important antioxidant-related mitochondrial enzyme systems include NADP-dependent isocitrate dehydrogenase, malic enzyme, and the $\Delta\mu_{H+}$ - dependent transhydrogenase. These enzymes are all important

in producing the NADPH used by the GR and transferase enzymes and in the thioredoxin and glutaredoxin reductase reactions.

Formation of Nitric Oxide in the Brain

NO is a unique signaling gas molecule that binds to the heme moiety of guanylyl cyclase, causing a conformational change, activating enzyme activity, and forming guanosine 3,5-cyclic monophosphate (cGMP). As a small gas molecule, it diffuses rapidly through cell lipid membranes, and therefore can be released from neuronal axons into the extracellular space as a potent nonreceptor-mediated signaling molecule among neurons.

NO is formed following activation of the enzyme nitric oxide synthase (NOS), which exists in three established isoforms in brain tissue, best classified by their dependency on Ca^{2+} for activation. The constitutive Ca^{2+}- dependent forms are endothelial NOS (eNOS) and neuronal NOS (nNOS), while the inducible Ca^{2+}-independent form is known as ‘‘iNOS.’’ Although named by the tissue in which they were originally discovered, these isoforms of NOS are not tissue specific. The exact contribution of each isoform to neurotoxicity and neuroprotection during brain injury is unknown. In general, results obtained both in vitro and in vivo suggest that eNOS activation with NO acting as a vasodilator is neuroprotective, while nNOS and iNOS are thought to be neurotoxic (Dalkara et al., 1998; Dirnagl et al., 1999). For example, during the initial stages of brain ischemia, activation of nNOS is associated with N-methyl-D-aspartate (NMDA) receptor activation while activation of iNOS occurs as a consequence of stimulated macrophages or astrocytes (including microglia), mainly during the reperfusion stage. This concept of neurotoxicity vs. neuroprotection, based on isoform activation, is probably too simplistic.

Mitochondrial Formation of NO

A number of investigators have reported that neuronal mitochondria produce NO in situ, catalyzed by NOS (Ghafourifar and Richter, 1999; Lacza et al., 2001). Prior to this discovery, it was believed that postsynaptic locations of nNOS, held by scaffolding proteins in close association with the NMDA receptor, represented the main sources for neuronal NO-related toxicity (Duchen et al., 2000). Much less is known, however, if mitochondrial formation of NO and/or $ONOO^-$ formation in situ plays a direct role in mediating the observed NO-related mitochondrial membrane changes or if it acts as an essential cell signal to mediate cell death or survival mechanisms (Radi et al., 2002). Mitochondria produce NO through a

Ca^{2+}-sensitive NOS identified in mitochondria isolated from liver, heart, kidney, and brain (Lacza et al., 2001; Aguilera-Aguirre et al., 2002). One hypothesis under investigation is that NOS located within neuronal mitochondria is activated during pathological conditions, e.g. as hypoxia/ischemia, in a Ca^{2+}-dependent manner. The proximity of the NO formed by mtNOS to mitochondrially generated superoxide would, in turn, result in $ONOO^-$ formation. Endogenous mitochondrial production of NO and its metabolites may therefore contribute to the oxidative modification of mitochondrial proteins. Evidence of intramitochondrial NO-mediated pathology includes the observation of parallel translocation of the mitochondrial-located proapoptotic factor CytC with nitrated proteins following brain oxygen–glucose deprivation (Alonso et al., 2002).

The physiological role of mtNOS in the cell is unknown. It has been demonstrated that mtNOS activity is functionally upregulated during hypoxia (Lacza et al., 2001). Some studies have led to the hypothesis that mitochondrial production of NO helps normalize O_2 utilization between cells at different distances from capillaries. The basic concept is that NO inhibits O_2 consumption by cells closest to the capillaries, allowing O_2 to penetrate to cells further away that would be severely hypoxic without buffering of the O_2 gradient. In this regard, mitochondrial NO together with other sources aids in dilation of blood vessels, thereby increasing cerebral blood flow and oxygenation of brain tissue at risk of becoming hypoxic (Haynes et al., 2003).

There is a paucity of data on whether intramitochondrial formation of NO and $ONOO^-$ is affected by the redox state, electrochemical properties of the mitochondria, its pH, or the ionic or enzymatic milieu. There is a need to more comprehensively understand the role of NO in mitochondrial damage and function during oxidative stress. The relationship of NO reactants formed within neuronal mitochondria to Ca^{2+} homeostasis and to mitochondrial respiration is currently not well defined.

Mitochondria as Targets of Reactive Oxygen and Nitrogen Species

It is well established that during chronic neurodegenerative diseases and following acute brain ischemia/injury, ROS and RNS contribute to mitochondrial damage (Brorson et al., 1999; Stewart and Heales, 2003). The origin of the ROS is multifactorial but includes mitochondria-generated superoxide (O_2^-), hydrogen peroxide (H_2O_2), NO radical, and its reactant anion, peroxynitrite ($ONOO^-$) (see figure 6). The ability of $ONOO^-$ to act as a neurotoxicant when generated at relatively high concentrations is well defined (Estevez et al., 1998). However, at lower, nanomolar concentrations, $ONOO^-$ is critical for triggering

neuroprotective signaling, including activation of antiapoptotic pathways (Garcia-Nogales et al., 2003; Bolanos et al., 2004). Importantly, although the majority of effects of $ONOO^-$ on brain tissue are deleterious, at low concentrations, there is evidence that $ONOO^-$ is neuroprotective (Garcia-Nogales et al., 2003).

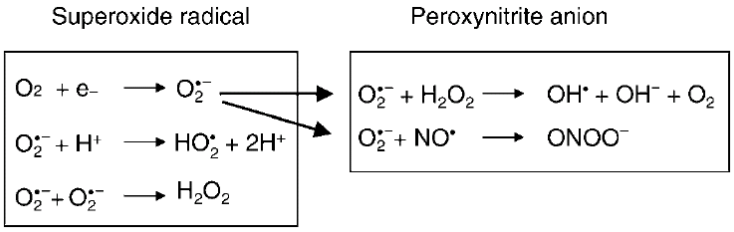

Fig.6. *Reactions forming reactive oxygen and nitrogen species. Adapted from L. Soaneet al., Mitochondrial Mechanisms of Oxidative Stress and Apoptosis (2007).*

NO, being a gas, can easily diffuse across biological membranes and enter mitochondria without requiring any receptor binding processes or transporting systems. In the presence of superoxide anion, $ONOO^-$ anion is formed. As an uncharged lipophilic molecule, NO contains a single unpaired electron (NO^-). NO can function either as an electron donor (oxidant) or as an electron acceptor (antioxidant). This property allows it to react with many types of molecules, including O_2, glutathione, and superoxide.

There is convincing evidence that ROS, NO, and its metabolites are toxic to mitochondria by damaging the ETC and the inner mitochondrial membrane in which the ETC resides (Moncada and Erusalimsky, 2002; Carreras et al., 2004). NO inhibits ETC activity by reversibly inhibiting complex IV (cytochrome oxidase, COX). During cerebral ischemia and hypoxia, the combination of reduced O_2 tension and elevated NO production might result in effective competitive inhibition of COX by NO, leading to impaired respiration and oxidative phosphorylation. Moreover, respiratory inhibition produces a reduced shift in the redox state of ETC components that, in turn, promotes the single electron reduction of O_2, forming superoxide. Elevated superoxide in the presence of elevated NO generates $ONOO^-$.

$ONOO^-$ can irreversibly inhibit many components of the ETC necessary for oxidative phosphorylation, including complexes I, II, and IV, and the ATP synthase (Brown and

Borutaite, 2004; Carreras et al., 2004) and a variety of RNS-mediated inhibitory mechanisms have been suggested. NO, via its effects on mitochondria, can induce necrosis or apoptosis, depending on the degree to which ATP levels are compromised (Bal-Price and Brown, 2000). In addition, both NO and $ONOO^-$ are capable of upregulating specific steps in glucose metabolism through their effects on gene expression (Cidad et al., 2004).

NO reactants can generate potent nitrating species leading to the formation of 3-nitrotyrosine in proteins. Two of the more commonly accepted in vivo mechanisms include the formation of either $ONOO^-$ or nitrogen dioxide. Both reactants are potent unstable species capable of interacting with proteins near the site of their generation. As illustrated in figure 6, $ONOO^-$ is formed by the interaction of superoxide radical with NO and is responsible for the oxidation, nitration, and nitrosylation of a variety of proteins (Jaffrey et al., 2001; Ischiropoulos and Gow, 2005). Nitration refers to the binding of a NO_2 group to a tyrosine or tryptophan residue, whereas nitrosylation refers to the binding of a NO group to a transition metal or cysteine residue (or other thiols) (Ischiropoulos et al., 2003; Gow et al., 2004). Nitration of tyrosine residues can have important activating and inhibitory effects on regulatory proteins since it prevents tyrosine phosphorylation. Nitrosylation of cysteines in general, plays a more important role in NO-mediated signal transduction. S-nitrosylation also inhibits the catalytic site of a variety of enzymes including some caspases, preventing inappropriate initiation of apoptosis (Kang et al., 2004). In contrast, nitrosylation of CytC promotes caspase activation (Cassina et al., 2000). As mentioned earlier, there are many mitochondrial proteins that undergo tyrosine nitration even under nonpathological conditions (Radi et al., 2002). Therefore, although protein nitration can interfere with mechanisms of cellular regulation and play a large role in mediating mitochondrial and neuronal toxicity, it may also play an important role in normal cell physiology (Aulak et al., 2004; Elfering et al., 2004).

Sildenafil and PDEs

Phosphodiesterase type-5 inhibitors (PDE5) are a new class of vasoactive drugs that have been developed for treatment of erectile dysfunction (ED) (Boolell et al., 1996; Porst et al., 2001). Their mechanism of action involves active inhibition of the PDE5 enzyme and resulting increase in cyclic guanosine monophosphate (cGMP) and smooth muscle relaxation in the penis.

There are 11 families of phosphodiesterases (PDEs) that have been identified in mammalian tissues (Corbin and Francis, 2002; Corbin et al., 2002). PDEs are found in all tissues, but the distribution of the various PDEs varies among different tissues and cell types (Wallis et al., 1999). PDEs contribute importantly to the modulation of diverse physiologic processes (Beavo et al., 1995). PDE5 is abundant in the corpus cavernosum and is the predominant PDE in this tissue (Gopal et al., 2001). Although other PDEs are present in the corpus cavernosum, they do not appear to significantly modulate changes in cGMP levels associated with the ability to achieve penile erection.

The PDEs vary in their substrate specificity for cyclic adenosine monophosphate (cAMP) and cGMP: PDE5, PDE6 and PDE9 are specific for cGMP; PDE4, PDE7 and PDE8 are specific for cAMP; and PDE1, PDE2, PDE3, PDE10 and PDE11 have mixed specificity for cAMP/cGMP (Rotella at al., 2002). Although PDE3 has similar affinity for cAMP and cGMP, it demonstrates lower enzymatic activity against cGMP. The PDE5 inhibitors compete with the substrate cGMP for binding to the protein at the catalytic site. Although cGMP binding to the catalytic site stimulates cyclic-nucleotide binding to the allosteric sites, inhibitors do not elicit the same property.

PDE5 is the primary enzyme with cGMP-hydrolysing activity in human corpus cavernosal tissue. Erection is largely a hemodynamic event, which is regulated by vascular tone and blood flow balance in the penis (Rotella et al., 2002). Because cGMP levels modulate vascular tone, it is an obvious target for therapeutic intervention in the process of erection. When a man is sexually stimulated, either physically or psychologically, nitric oxide (NO) is released from non-cholinergic, non-adrenergic neurons in the penis, as well as from endothelial cells. NO diffuses into cells, where it activates soluble guanylyl cyclase (GC), the enzyme that converts GTP to cGMP. The cyclic nucleotide then stimulates protein kinase G (PKG), which initiates a protein phosphorylation cascade. This results in a decrease

in intracellular levels of calcium ions, leading ultimately to dilation of the arteries that bring blood to the penis and compression of the spongy corpus cavernosum. A PDE5 inhibitor blocks enzymatic hydrolysis of cGMP in the human corpus cavernosum, leading to the same outcome (see figure 7).

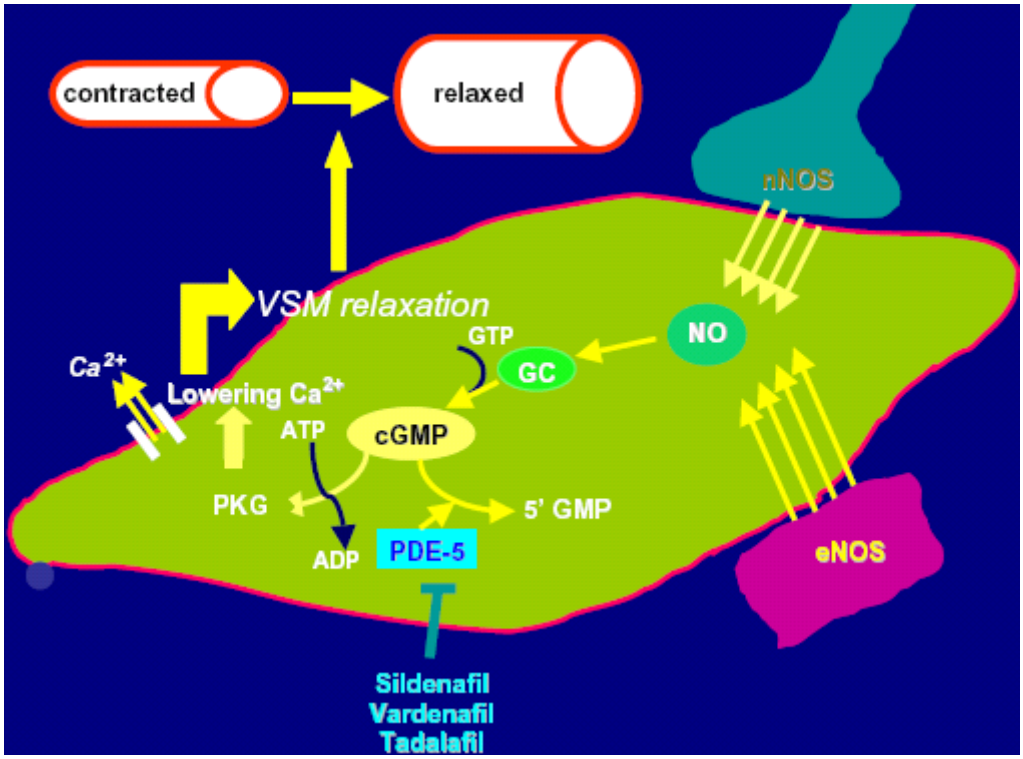

Fig.7. *Mechanism of penile erection: sexually stimulation releases NO from non-cholinergic, non-adrenergic neurons in the penis, as well as from endothelial cells. NO diffuses into cells and activates soluble guanylyl cyclase, which converts GTP to cGMP. The cyclic nucleotide then stimulates protein kinase G (PKG), which initiates a protein phosphorylation cascade, thereby decreasing intracellular levels of calcium ions, leading ultimately to dilation of the arteries that bring blood to the penis and compression of the spongy corpus cavernosum. Phosphodiesterase type-5 (PDE5) is the target for sildenafil and other PDE5inhibitors in the treatment of erectile dysfunction. GTP=guanosine triphosphate, PKG=cGMP-dependent protein kinase, VSM=vascular smooth muscle. Adapted from R.C. Kukreja et al. / Vascular Pharmacology 42 (2005) 219–232.*

Sildenafil citrate (Viagra^{TM}) is the first PDE5 inhibitor approved for treatment of erectil dysfunction (ED). The discovery of this drug in 1989 was the result of extensive research on chemical agents that might potentially be useful in the treatment of coronary heart disease. Initial clinical studies on sildenafil in the early 1990s were not promising with respect to its antianginal potential. However, a remarkable side effect was reported by a number of

volunteers participating in these investigations; sildenafil seemed to enhance penile erections, which soon thereafter became the main focus of further studies. More than 10 million men worldwide have been treated with sildenafil since its market debut in 1998. It is highly specific for PDE5 inhibition with relatively minor cross-reactivity with PDE6 (Laties and Fraunfelder, 1999). It has a chemical structure similar to cGMP (see figure 8) and inhibits PDE5 by binding to the cGMP-catalytic sites (Corbin and Francis, 2002) thereby allowing the accumulation of cGMP in erectile tissue.

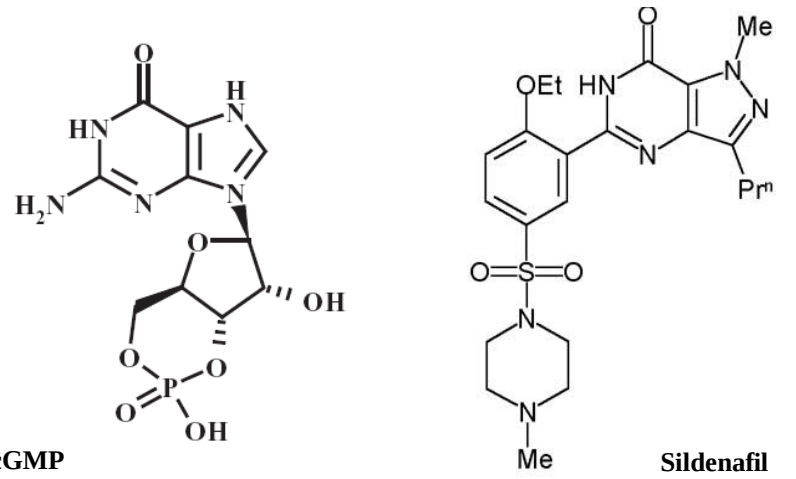

Fig.8. *Comparison of the structure of PDE5 inhibitor, sildenafil, with the native substrate cGMP. Adapted from R.C. Kukreja et al. / Journal of Molecular and Cellular Cardiology 36 (2004) 165–173.*

It was recognized that NO serves as a neurotransmitter/neuromodulator in the central and peripheral nervous systems and that certain neural cells possess a cGMP signaling pathway similar to that in vascular smooth muscle cells. Although NO (at high concentrations) is toxic and thought to participate in neuronal cell death during stroke and neurodegenerative diseases, recent evidence suggests that NO at low physiological concentrations can act as an antiapoptotic/prosurvival factor in certain neural cells. The antiapoptotic effects of NO are mediated, in part, by cGMP and a downstream target protein, PKG, as mentioned above in the chapter ´ Formation of Nitric Oxide in the Brain ´.

Huntington's disease (HD) is an autosomal dominant disease affecting the central nervous system (CNS) that leads to the dysfunction and death of neuronal cells in the brain. The disease is characterized by a loss of cognitive ability and motor skills, eventually leading to dementia and changes in personality, as well as severe movement dysfunction known as chorea and to eventual death (Lindsay et al., 2006). Selective neuronal loss, predominantly in the striatum and other basal ganglia structures, accounts for the majority of the clinical features of Huntington's disease. But the exact mechanisms underlying neuronal death in Huntington's disease are unknown.

It is clear that impairment of mitochondrial energy metabolism is the key pathogenic factor in a number of neurodegenerative disorders (Schon and Manfredi, 2003). Mitochondria have been proposed to be the main source of reactive oxygen species (ROS) in neuronal cells. Cells contain antioxidant systems to block ROS overproduction, including glutathione (GSH) and nicotinamide adenine dinucleotide coenzyme (NADH / NAD^+) and its derivatives (NADPH / $NADP^+$) (NAD(P)H), which play a crucial role as part of the primary cellular defence against oxidative stress. GSH depletion has been reported after different apoptotic stimuli including 6-OHDA (Galindo et al., 2004), veratridine (Jordan et al., 2002), MPTP (Selley, 1998) or malonate (Ehrhart & Zeevalk, 2003).

Toxins that affect mitochondria are being used as pharmacological tools to mimic several of these diseases. Among others, 3-NP, MPTP, rotenone and malonate are well-established mitochondrial complex inhibitors frequently used to investigate the key cellular pathways that provoke neurodegeneration in Parkinson's or Huntington's diseases (Browne & Beal, 2002).

Malonate has been shown to cause dose-dependent neurotoxicity both 'in vivo' and 'in vitro' by inhibition of succinate dehydrogenase and depletion of striatal ATP (Stokes et al., 2001, Van Westerlaak et al., 2001) resulting in neuronal depolarization and secondary excitotoxicity (Henshaw et al., 1994; Greene and Greenamyre, 1996). It was suggested that malonate toxicity involves neurons dying not only by necrosis but also by delayed caspase activation and apoptosis (Schulz et al., 1998).

Consistent with the well-known inhibitory effect on mitochondrial complex II, some reports demonstrate that malonate induces cell death through a mechanism that involves a direct participation of the mitochondria. Malonate induces a rapid depolarization of the mitochondrial electric potential and a delayed swelling of the organelle. Malonate increased the rate of ROS formation in mitochondria resulting in the depletion of ROS scavenger systems including GSH and NAD(P)H levels (Lindsay et al., 2006). Many reports have been published, but the exact mechanism by which malonate induces toxicity remains still unclear.

Interestingly, the attenuation of ROS production ameliorated the effect of malonate on p38 MAP kinase phosphorylation, suggesting that malonate promotes ROS production which subsequently activates p38 MAP kinase (Ghatan et al., 2000; Choi et al., 2004). In our study, the p38 MAP kinase inhibitor, SB503580, proves the participation of this MAPK signalling pathway in the mechanisms underlying malonate-induced neurototoxicity. We deduce this from the significant attenuation of striatal malonate-induced striatal lesion after SB203580 administration. Indeed, there is a significant body of evidence suggesting that p38 MAP kinase activation plays an important role during excitototoxic and neurodegenerative processes (Cao et al., 2004).

Moreover, malonate produced a marked increase in the phosphorylation of p38 MAP kinase in SH-SY5Y cells (Jordan et al., 2006). According to these results we tried to evaluate p38 MAP kinase activation in vivo using specific antibodies for this protein. However we could not find the ideal conditions of Western immunoblotting analysis to give a confirmative and steady results showing phosphorylated p38 MAP kinase.

Furthermore, use of the p38 MAP kinase inhibitor, SKF86002, potently inhibited Bax translocation and offered significant protection against malonate-induced apoptosis in vitro (Jordan et al., 2006). The pro-apoptotic action of Bax is believed to be mediated by its insertion into the outer mitochondrial membrane where it might directly form channels or regulate the activity of pre-existing channels (Sharpe et al., 2004). This process triggers the release of intermembrane space proteins into the cytoplasm, including cytochrome c. Today it is an established fact that cytochrome c, once present in the cytosol, drives the assembly of a high

molecular weight caspase activating complex termed the ‘apoptosome’, which contributes to apoptosis.

There is an evidence that malonate-induced apoptosis of SHSY5Y neuroblastoma cells is mediated via the pro-apoptotic Bcl-2 family protein Bax (Jordan et al.,2006). We therefore tried to elucidate whether the same chain of biochemical events reported in vitro after malonate also take place in our in vivo model.

We provide a converse evidence that neither Bax translocation into the mitochondria nor consecutive release of cytochrome c into the cytosol participate in malonate-induced neurotoxicity of the rat striatum. There are no significant changes between Bax levels in the mitochondria and in the cytosol. Neither any cytochrome c is present in the cytosol after malonate injection into the rat striatum.

We also prove that reactive oxygen species are generated in malonate-induced lesion of rat brains. Ethidium fluorescence, thus superoxide and superoxide-derived oxidant production, increased in rat striata injected with malonate. Using fluorescence microscopy we demonstrate photos of neural tissue presenting this phenomena in our work.

It is also well established that several human neurodegenerative disorders, including stroke, Alzheimer’s disease (AD), Parkinson’s disease (PD) and Huntington's disease are accompanied by elevated oxidative stress. More specifically, reactive oxygen species (ROS) have been demonstrated to mediate neuronal death in these neurodegenerative diseases. The extent of delayed neuronal death correlates well with prelethal markers of oxidative molecular alterations to lipids, proteins, and nucleic acids in both animal models and patients (Cristofori et al., 2005). These oxidative modifications are also observed at the mitochondrial level (Naoi et al., 2005), including e.g. possible participation of UCPs, mitochondrial ATP-sensitive potassium channels, mitochondrial Ca^{2+} production etc. Although we have not been able to detect phosphorylated p38 MAPK in vivo, the idea, that malonate activates ROS which subsequently cause p38 MAP kinase phosphorylation, seems to be one of the possible eventualities.

We have tried to find some of the possible signalling pathways, which could contribute to malonate-induced neuron death. We confirm that ROS and p38 MAP kinase participate in this process, however we found no evidence for Bax translocation into the mitochondria and cytochrome c release into the cytosol at various times after malonate injection.

At the beginning of this study we provide obvious evidence, that sildenafil causes a large neuroprotection against malonate-induced lesion of the rat striatum. We also prove that sildenafil does not have any effect on ROS production, which appear in rat striatum after malonate injection. Obviously, we have not been able to elucidated the signalling pathways by which sildenafil causes neuroprotection against malonate in this work. The mechanisms underlying malonate-induced cell death, increased production of reactive oxygen species, the participation of several apoptotic pathways and the involvement of different signalling pathways seems to be very complex and remains unclear so far.

Anyway, the neuroprotection afforded by sildenafil was so large, as photos of the rat brain slices show, that it diserves further scientific investigation. Signalling pathways activated either by modulating the accumulation of ROS or by pharmacological inhibition of p38 MAP kinase may afford protection against malonate toxicity.

These findings might have important therapeutic implications for the treatment of neurodegenerative disorders such as Huntington's disease and neuroprotective effects of sildenafil could be a great promise for the future.

1. Sildenafil administered both 30 minutes or 24 hours before striatal stereotaxic injection of malonate provides a large neuroprotective effect in rat brains. Sildenafil significantly decreases the lesion volume in the rat striatum caused by malonate.

2. The administration of SB203580, a selective inhibitor of p38 MAP kinase, provides a significant protection against malonate-induced neurotoxicity. These data suggest that the p38 MAP kinase signalling pathway plays an important role in the mechanisms underlying malonate toxicity.

3. The protective effects of sildenafil cannot be attributed to any effect on ROS production. Thus, hydroethidine oxidation was similar in rats treated with malonate alone or in combination with sildenafil.

4. No evidence of Bax redistribution from the cytosol into the mitochondria was found after malonate intoxication. Bax levels remained unchanged both in the mitochondria and cytosol of malonate-treated rats. There are no significant changes of Bax levels among malonate administered animals and control group.

5. Cytochrome c release from the mitochondria to the cytosol does not occur after malonate administration. Cytochrome c levels analysed in the mitochondria did not differ significantly between malonate-treated rat and the control group. Furthemore, the presence of cytC in the cytosol was not detected at any time after malonate injection.

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