Mitochondria Play a Central Role in Estrogen-Induced Neuroprotection

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Abstract: Oxidative stress, bioenergetic impairment and mitochondrial failure have all been implicated in the etiology of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD), as well as retinal degeneration in glaucoma and retinitis pigmentosa. Moreover, at least 75 debilitating, and often lethal, diseases are directly attributable to deletions or mutations in mitochondrial DNA, or in nuclear-encoded proteins destined for delivery to the mitochondria. Such widespread mitochondrial involvement in disease reflects the regulatory position mitochondrial failure plays in both acute necrotic cell death, and in the less catastrophic process of apoptosis. The potent feminizing hormone, 17 β-estradiol (E2), has shown cytoprotective activities in a host of cell and animal models of stroke, myocardial infarct and neurodegenerative diseases. The discovery that 17·α-estradiol, an isomer of E2, is equally as cytoprotective as E2 yet is >200-fold less active as a hormone, has permitted development of novel, more potent analogs where cytoprotection is independent of hormonal potency. Studies of structure-activity-relationships, glutathione interactions and mitochondrial function have led to a mechanistic model in which these steroidal phenols intercalate into cell membranes where they block lipid peroxidation reactions, and are in turn recycled via glutathione. Such a mechanism would be particularly germane in mitochondria where function is directly dependent on the impermeability of the inner membrane, and where glutathione levels are maintained at extraordinarily high 8-10mM concentrations. Indeed, the parental estrogens and novel analogs stabilize mitochondria under Ca^{2+} loading otherwise sufficient to collapse membrane potential. The cytoprotective and mitoprotective potencies for 14 of these analogs are significantly correlated, suggesting that these compounds prevent cell death in large measure by maintaining functionally intact mitochondria. This therapeutic strategy is germane not only to sudden mitochondrial failure in acute circumstances, such as during a stroke or myocardial infarction, but also to gradual mitochondrial dysfunction associated with chronic degenerative disorders such as AD, PD and HD.

Keywords: Mitochondria, estrogens, neurodegeneration, neuroprotection, apoptosis, necrosis, calcium homeostasis, mitochondrial membrane potential, reactive oxygen species.

1. INTRODUCTION

Mitochondria are increasingly recognized as subcellular organelles that are essential for generating the energy that fuels normal cellular function, yet at the same time are the major intracellular source of cytotoxic free radicals and the primary determinants of cell death [1,2]. The unique role of the mitochondria is in supplying high energy ATP molecules, while at the same time, monitoring cellular health in order to make a rapid decision to initiate a programmed cell death program. As such, the mitochondria sit at a strategic position in the hierarchy of cellular organelles to continue the healthy life of the cell or to terminate it.

Nowhere is this critical role of mitochondria more important than in neurons of the central nervous system. The brain has a high energy demand: although the brain represents only 2% of the body weight, it receives 15% of cardiac output and accounts for 20% of total body oxygen consumption. This energy requirement is largely driven by neuronal demand for energy to maintain ion gradients across the plasma membrane that are critical for the generation of action potentials. This intense energy requirement is continuous; even brief periods of oxygen or glucose deprivation result in neuronal death.

In view of these considerations, it is not unexpected that the role of the mitochondria in neurological diseases, particularly those that have a neurodegenerative component, is now the subject of much research.

2. NEUROLOGICAL DISEASES WITH A MITOCHONDRIAL ETIOLOGY

Of the over 75 diseases associated with mitochondria dysfunction, many are attributable to mutations or deletions in the mitochondrial DNA (mtDNA), an intronless, circular genome of 16.5 kB encoding 37 genes, 13 of which are for proteins that serve as active sites in the electron transport system, with the remainder encoding for elements required

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for expression. Mitochondriopathies typically have a chronic, slowly progressive course with multiorgan involvement [3]. Of the approximately 3000 proteins in the mitochondrial proteome, most mitochondriopathies are associated with deficits in the electron transport system that increase free radical production while at the same time repress energy production. Organ systems at particular risk in mitochondriopathies are aerobically poised, long-lived and metabolically active tissues, such as the peripheral and central nervous systems, where dementias, epilepsy, and ataxias are frequently present, the eyes, where glaucoma, retinopathy, and optic atrophy occur, and the heart. In addition, deafness, muscular weakness, diabetes, renal and bone marrow failure often complicate these disorders [4]. For example, the 3243A>G mutation is one of the most frequently observed mutations of mtDNA, and is associated with a several clinical presentations including mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), progressive external ophthalmoplegia (PEO) and diabetes [5,6]. Mutations in mtDNA have also been identified in other subgroups of mitochondrial encephalomyopathies, e.g., point mutations in mitochondrial tRNA-lys in MERRF syndrome (myoclonus epilepsy associated with ragged-red fibers) as well as in Leber's heredity optic neuropathy (LHON) [7], which presents in mid-life as acute loss of central vision leading to blindness.

Other diseases caused by mitochondrial failure are due to mutations in nuclear genes encoding proteins that are imported into the mitochondria. For example, Friedreich's ataxia is a recessively inherited early onset disease that tragically affects children between 5 and 15 years old. It is characterized by progressive deterioration of the CNS, resulting in debilitating muscle weakness and heart disease, and most patients die in early adulthood. Friedreich's ataxia is caused by large expansions of a GAA repeat in the first intron of the gene for the protein called frataxin [8]. Frataxin is involved in iron homeostasis, and these repeats impede its translocation into the mitochondria, causing excessive iron availability within mitochondria. Iron is one of several transition metals that can serve as Fenton catalysts to accelerate production of .OH from H_2O_2. Because of redox potentials, any of the electron transport components associated with the mitochondrial inner membrane has the capacity to reduce molecular oxygen univalently to superoxide. Although superoxide is fairly reactive, especially towards Fe-S centers that serve as the active sites in a host of mitochondrial electron transport proteins and enzymes like aconitase proteins, it is hydroxyl radical that is particularly damaging to the membrane integrity that underlies mitochondrial function [9,10]. Excessive iron in FRDA mitochondria exacerbates oxidative stress, and especially promotes membrane lipid peroxidation reactions that can directly undermine mitochondrial function by degrading the impermeability of the inner membrane [11]. It is for this reason that membrane-selective antioxidant strategies, like those shown by the estrogen-like, polyphenolic compounds (PPCs), may well provide therapeutic benefit in FRDA.

Other chronic diseases with bioenergetic and oxidative etiologies that implicate mitochondrial dysfunction include Alzheimer's and Parkinson's diseases, as well as amyotrophic lateral sclerosis, (ALS) where a mutation in superoxide dismutase is associated with the familial disease [12].

Although the etiology of AD is multifactoral, [13], a consistent finding is hypometabolism of glucose in only those brain regions affected by the disease [14] that can be detected very early in the disease, even before cognitive symptoms are reported [15,16]. Although this might be construed as simply reflecting neuronal loss in the effected regions, mitochondrial dysfunction can be more directly implicated in AD not only because, β-amyloid undermines mitochondrial stability, inducing both oxidative and bioenergetic crises, [17-19], but also because such mitochondrial impairment in turn enhances the production of Aβ [20]. Similarly, mitochondrial dislocation, resulting from disruption of normal axonal transport of mitochondria due to the breakdown of microtubules from the hyperphosphorylation of the microtubule-associated protein, tau, could participate in AD progression secondarily to mitochondrial failure [21,22]. In any event, mitochondria from AD subjects are hypofunctional [14,23], and produce excessive reactive oxygen species (ROS) [24,25]. A catalytic defect in respiratory complex IV (C-IV) has been described in mitochondria from AD subjects [26,27], and when inserted into transformed cells depleted of their endogenous mtDNA, mtDNA from AD patients produce a phenotype in the resulting cytoplasmic hybrids (cybrids), of increased oxidative stress, propensity towards apoptosis, and C-IV impairment [28,29], suggesting that many of the cellular defects found in AD reflect mitochondrial defects. Although such mitochondrial impairment could be interpreted as a consequence of the disease, not as a primary causal factor [30], mitochondrial dysfunction is clearly involved in progressive neuronal death and as such represents a viable therapeutic target.

The evidence for mitochondrial defects in Parkinson's Disease (PD) is more consistent, with impairment in respiratory complex I (C-I) documented from brain and peripheral tissues [31-33]. Cybrids containing mtDNA from PD patients not only show comparable mitochondrial impairment in complex I activity and ensuing oxidative stress [34], but also Lewey bodies in these cybrids react positively with cytochrome c antibodies suggesting a mitochondrial origin [35]. Mitochondrial involvement in the etiology of PD is also supported by toxin models where selective inhibitors of respiratory C-I, such as MPTP, rotenone and 6-OH-dopamine, produce substantia nigral degeneration and PD symptoms in animals and humans [36,37]. Indeed, clinical trials with coenzyme Q10 to treat PD patients are already showing some promise [38].

Insights into the involvement of mitochondrial senescence in Huntington's Disease have paralleled those for PD, with oxidative stress, bioenergetic impairment and mitochondrial failure all contributing to pathology [39,40]. As is the case with PD, animal models of the disease are generated using a selective mitochondrial toxin, in this case a Complex II inhibitor, 3-nitropropionic acid, that yields specific behavioral changes and selective striatal lesions in rats and non-human primates that mimic those in HD [41,42].

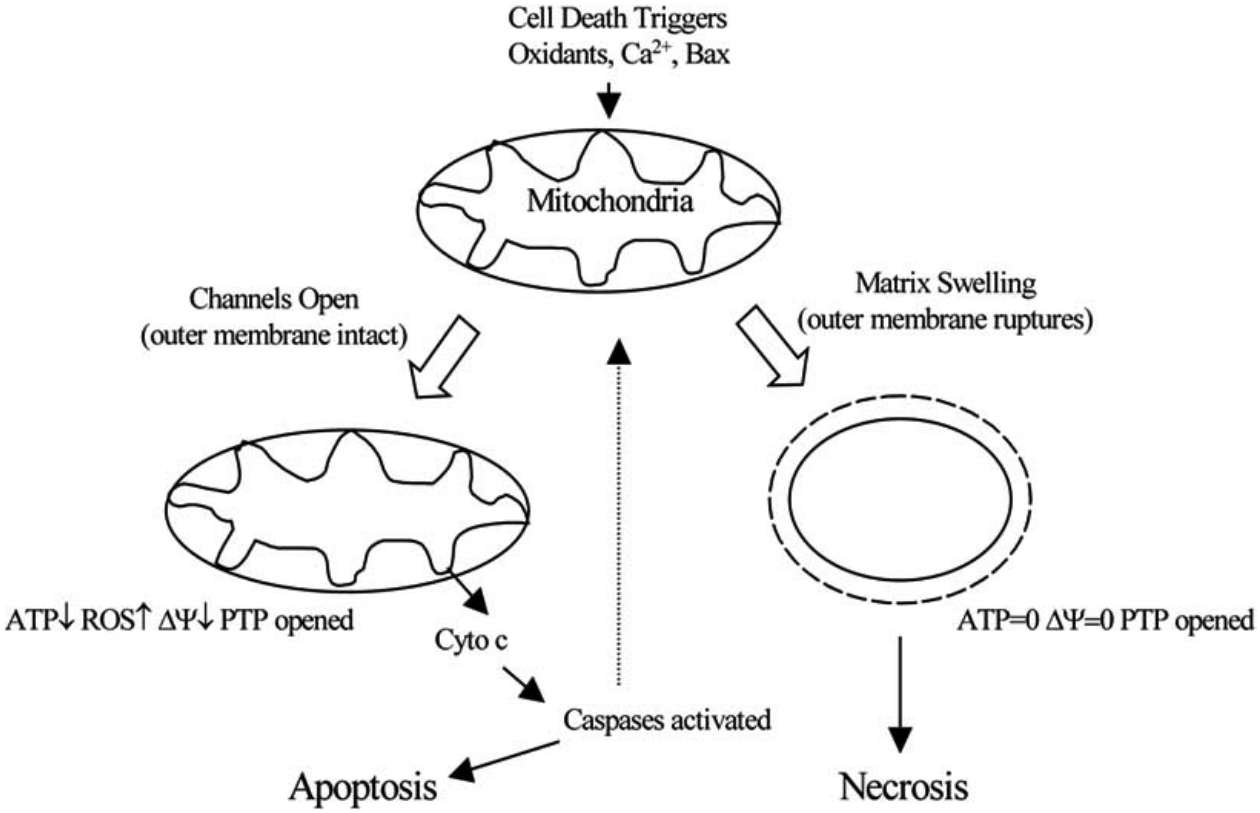

Fig. (1). Scheme of the involvement of mitochondria in cell death. A variety of stimuli, including pro-oxidants, Ca^{2+} overload, trophic factor withdrawal, pro-oxidant stress, can trigger cell death. Two general mechanisms have been proposed: one involves osmotic disequilibrium leading to an expansion of the matrix space, swelling of the mitochondria and rupture of the outer membrane, a process accompanied by complete energy breakdown resulting in necrosis (right); the other involves opening of channels in the outer membrane allowing mitochondria to release caspase-activating proteins, cytochrome c, apoptosis inducing factor (AIF) and intramitochondrial caspases into the cytosol. The release of caspase-activating proteins can further activate caspases and initiate the proteolytic cascade that culminates in apoptosis (left). Abbreviations used: ATP, adenosine triphosphate; ROS, reactive oxygen species; Cyto c, cytochrome c; PTP, permeability transition pore; ΔΨm, mitochondrial membrane potential; Ca^{++}, calcium.

3. MITOCHONDRIA AND NEURONAL DEATH

The above discussion focuses on chronic, slowly progressing pathologies, yet mitochondrial failure also contributes to cell death in more acute circumstances, such as sudden ischemia of neurons in a stroke, or of the myocardium during a heart attack. Neurons are dependent almost entirely on mitochondria ATP production for their high energy demand, and are at risk when ATP levels drop, even transiently. Damage to mitochondria causes disruptions in ATP production and a concomitant increase in ROS that can overwhelm the antioxidant defense systems of the cell [1,49]. Such mitochondrial deficits are implicated as key events in the pathogenic cascades leading to both necrosis and apoptosis [43,44]. Oxidative stress, coupled with excessive Ca^{2+} loading, cause mitochondria to undergo a catastrophic loss of the impermeability of the inner mitochondrial membrane that causes a collapse of the mitochondrial membrane potential (Δψm), a process called permeability transition (PT) [2]. This collapse of Δψm can be accompanied by mitochondrial swelling and release of cyotchrome c and Apaf-1 [45] into the cytoplasm where they activates caspases and induced apoptotic cell death [44,46]. This process undermines cellular and mitochondrial integrity by causing membrane peroxidation and interfering with oxidative phosphorylation by inactivating Fe-S clusters needed for the electron transfer system. The resulting loss of ATP production causes the ATPase failure, loss of ion homeostasis and necrosis from osmotic failure [1,47] (Fig. **1**).

Mitochondrial failure initiates apoptosis in response to a variety of stressors, including withdrawal of neurotrophic support, injury and exposure to excitatory amino acids, such as glutamate (Fig. **1**). Excessive Ca^{2+} from glutamate receptor activation or other Ca^{2+} permeable channels can lead to mitochondrial Ca^{2+} loading, interruption of ATP production, generation of ROS and loss of collapse of the Δψm, ultimately leading to neuronal death [48,49]. As such, mitochondria are believed to be the key modulator of neuronal viability during excitotoxicity [1].

4. MITOCHONDRIA PHYSIOLOGY AND PATHOLOGY

4.a. Mitochondrial Electron Transport

Mitochondria carry out most cellular oxidations and produce ATP. Large amounts of NADH and $FADH_2$ are produced by oxidation reactions that convert pyruvate and fatty acids to acetyl CoA and that oxidize the acetyl CoA to CO_2 through the citric acid cycle. The energy available from

combining oxygen with the reactive electrons carried by NADH and $FADH_2$ is harnessed by an electron-transport chain in the mitochondrial inner membrane. Protons are pumped by the electron transport chain out of the matrix to create a transmembrane electrochemical proton (H^+) gradient which includes both an electrical ($\Delta\Psi m$) and a chemical component (ΔpH). Under resting conditions, $\Delta\Psi m$ is approximately 170 mV and the pH gradient is approximately 0.5 pH units (alkaline inside). The transmembrane gradient in turn is used both to synthesize ATP by ATP synthase (termed oxidative phosphorylation) and to drive the active transport of selected metabolites across the mitochondrial inner membrane.

The inhibition of electron transport of neurons compromises their ability to generate an electrochemical gradient and, therefore, decreases the rate of ATP synthesis. In addition, mitochondrial free radical generation may be enhanced. Ultimately, either apoptotic or necrotic death, or chronic cellular dysfunction may result (Fig. **1**).

4.b. Mitochondrial Reactive Oxygen Species Generation

Reactive oxygen species (ROS) include hydroxyl radicals, superoxide anion, hydrogen peroxide and nitric oxide. The cytosolic enzyme family of NADPH oxidases (a superoxide-generating system) can contribute to ROS production. However, the majority of intracellular ROS production is derived from the mitochondria. Severe mitochondrial respiratory inhibition and excessive Ca^{2+} accumulation potentiates mitochondrial ROS production [49].

A rise in intracellular ROS levels has two potentially important effects: (1) triggering the activation of specific signaling pathways; and (2) damaging various cell components by oxidizing nucleic acids, proteins, and membrane lipids. The major aspects of cellular and molecular responses include alterations in the gene expression of antioxidant enzymes, stress-response genes, and cytokines. The regulatory mechanisms that control this genetic response are complex and may involve stimulation of signal transduction components such as Ca^{2+}-signaling and protein phosphorylation, and activation of transcription factors [50,51]. Oxidants such as superoxide, hydrogen peroxide, hydroxyl radicals, nitric oxide and lipid hydroperoxides are now recognized as signaling molecules under subtoxic conditions. The main signaling pathways in response to oxidative stress include the extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signaling cascades, the phosphoinositide 3-kinase (PI(3)K)/Akt pathway, the nuclear factor NF kappa B (NFκB), AP-1 signaling system, p53 activation and the heat shock response [52].

Consequences of oxidative stress include modification to cellular proteins, lipids and DNA. Carbonyl formation can occur through a variety of mechanisms including direct oxidation of certain amino-acid side chains and oxidation-induced peptide cleavage. Mitochondrial DNA is more sensitive than nuclear DNA to oxidative damage due to its proximity to the main source of oxidant generation or because of a limited mitochondrial DNA repair system [52]. When oxidative stress is severe, survival is dependent on the ability of the cell to adapt to or resist the stress, and to repair or replace the damaged molecules. Alternatively, cells may respond to the insult by undergoing cell death.

Cellular antioxidant defense systems include the enzymatic scavengers, superoxide dismutase (SOD), catalase and glutathione peroxidase. SOD promotes the conversion of superoxide to hydrogen peroxide, whereas catalase and glutathione peroxidase convert hydrogen peroxide to water. In addition to these well characterized antioxidant enzymes, at least five members of a new family of peroxide scavengers termed peroxiredoxins (Prxs) [53] have recently been isolated. Prxs are capable of protecting cells from ROS insult and regulating the signal transduction pathways that utilize caspases, NFκB and activator protein-1 (AP-1) to influence cell growth and apoptosis. A variety of other non-enzymatic, low molecular mass molecules are important in scavenging ROS. These include ascorbate, pyruvate, vitamine E, flavonoids, carotenoids and perhaps most importantly glutathione, which is present in millimolar concentrations within cells.

4.c. Mitochondrial Ca^{2+} Transport

Mitochondrial Ca^{2+} sequestration may contribute to cell death in excitotoxicity, ischemia reperfusion injury and neurodegenerative diseases [1,49,54]. Mitochondrial calcium loading depends on uptake through the uniporter and efflux by $Na+/Ca2+$ exchanger on mitochondrial membrane [55,56]. Calcium efflux is through the Na^+-Ca^{2+} exchanger which is influenced by the Na^+/H^+ antiporter that is driven by ΔpH, and the Na+ independent Ca^{2+} transporter [1,57]. Functions of mitochondrial Ca^{2+} transport including control of the metabolic rate for cellular energy (ATP) production, modulation of cytosolic calcium signaling, and activation of apoptosis through release of cytochrome c depends upon the degree of mitochondrial Ca^{2+} loading. Mitochondria take up and accumulate Ca^{2+} during physiological calcium signaling [58]. Low levels of mitochondrial Ca^{2+} sequestration, for example, following normal depolarization, can stimulate matrix Ca^{2+}-sensitive dehydrogenase activity which enhances ATP synthesis to meet the increased cellular energy demand. Higher levels of sequestration induce Ca^{2+}-induced Ca^{2+} release, which may play an important role in the propagation of Ca^{2+} signals. Above a certain level, Ca^{2+} sequestration can begin to compromise mitochondrial function, resulting in respiratory inhibition or uncoupling of oxidative phosphorylation, a drop in $\Delta\Psi m$, formation of nonspecific pores, or osmotic lysis [1,54].

The combination of mitochondrial Ca^{2+} uptake with oxidative stress may open the mitochondrial permeability transition pore (PTP), which will initiate pathways to cell death either by necrotic or apoptotic pathways [59]. However, many models of apoptosis do not require pore opening to cause cytochrome c release [60]. Nevertheless, it is accepted that mitochondrial calcium plays an important role in mitochondrial participation in cell death through its role in inducing the PTP or other undefined pathways [58,61].

4.d. Mitochondrial Permeability Transition

The mitochondrial permeability transition (MPT) is a sudden increase of the inner membrane permeability to

solutes with molecular mass below approximately 1500 Da (including Ca^{2+}, glutathione, pyridine nucleotides) to equilibrate across the membrane [62-64]. This phenomenon is readily induced by matrix accumulation of high levels of Ca^{2+}, and it is believed to be caused by the opening of a non-specific, voltage-dependent, cyclosporin A-sensitive, high-conductance inner membrane channel [65], although other models also have support [2]. The primary consequence of pore opening *in vitro* is the $\Delta\Psi m$ depolarization. Pore opening is always followed by depolarization, but depolarization is not always caused by pore opening [66]. Opening of this pore dissipates the H^+ gradient across the inner membrane and uncouples the respiratory chain. More importantly, PTP opening results in a volume dysregulation of mitochondria due to the hyperosmolality of matrix, which causes the matrix space to expand. This matrix volume expansion can eventually cause outer membrane rupture, resulting in necrotic cell death [2,67].

4.e. Bcl-2 Family Proteins

Bcl-2 family proteins are key regulators of apoptosis, acting to either inhibit or promote cell death. The major anti-apoptotic members of the bcl-2 family, bcl-2 and bcl-xL, reside on the mitochondrial outer membrane, the endoplasmic reticulum and perinuclear membrane [68]. Bcl-2 directly or indirectly prevents the release of cytochrome c from mitochondria [45,69]. Bax- and Bax-like proteins are cytosolic before an apoptotic stimulus [70]. In response to a death signal, their translocation, mitochondrial membrane insertion and homodimerization can result in cell death [71].

5. NEUROPROTECTIVE EFFECTS OF ESTROGENS

Epidemiological studies have shown that estrogen therapy (ET) soon after the menopause is associated with numerous beneficial health effects including a reduction in risk for cardiovascular diseases, decreased incidence of osteoporosis and associated bone fractures [72,73], decreased risk for neurodegenerative diseases [74], increased cognitive performance [75], and reduced risk for cataract [76-78].

There is plethora evidence from *in vitro*, *in vivo* and clinical studies show that estrogen is protective against various oxidative stress insults including serum deprivation [79-82], amyloid β peptide (Aβ)-induced toxicity [83-92], glutamate-induced excitotoxicity [85,93-96], hydrogen peroxide (H_2O_2) [83-85, 97-99], oxygen-glucose deprivation (OGD) [100-101], iron [85,102], hemoglobin [100] and mitochondria toxins such as 3-NPA [103], N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [104] and sodium azide [100].

The neuroprotective effects of estrogens have been demonstrated in a variety of models of acute cerebral ischemia. These include transient and permanent middle cerebral artery occlusion models [105-107], global forebrain ischemia models [108,109], photothrombotic focal ischemia models [110], and glutamate-induced focal cerebral ischemia models [111]. The protective effects of estrogens have been described in rats, mice and gerbils [112,113]. Estrogen-induced neuroprotection has been demonstrated in adult female, middle-aged female as well as reproductively senescent female rats [114]. Similarly, these effects of estrogens have been shown despite the presence of diabetes and hypertension [115,116]. The neuroprotective effects of estrogens have been demonstrated against subarachnoid hemorrhage, a highly prevalent form of stroke in females [117]. Finally, the neuroprotective action of estrogen is not limited to the female, inasmuch as estrogen protection is also seen in males [118,119]. Collectively, these results indicate that estrogens could be valuable candidates for brain protection during acute stroke in both males and females.

The concentrations of estrogens, ranging from low physiological to high pharmacological, have been shown to produce protective effects in stroke models. No neuroprotection was afforded by the administration of physiological level of estradiol at the onset of an ischemic event [107], but neuroprotective effects of pharmacological doses of estradiol were clearly demonstrated by the acute treatment at the time of or just before an ischemic event, as well as after its onset [105,120-122]. The therapeutic window of estrogens at the dose of 100μg/kg lasts up to 3 hours after insult [121] and this therapeutic window can be extended to up to 6 hours after ischemic insult with doses of 500 to 1,000 μg/kg [122]. This long post-event efficacy of estrogens is promising, since the therapeutic window for estrogen neuroprotection could be insult severity-dependent and could be different between different species. It has been shown that the infarct penumbra, which is can be protected, develops over a longer period in human subjects than in rodents [123]. So, it is reasonable to predict that estrogens could have a longer therapeutic window in human than the 6 hour window that we have described in rodents.

6. MITOCHONDRIAL EFFECTS OF ESTROGENS

The possibility that estrogens exert their potent neuroprotective effects through a mitochondrial mechanism is based on several observations. First, estrogen binding sites have been described in the mitochondria, including the F0/F1 ATPase [124,125] and we have demonstrated that the estrogen receptor (ER) beta localizes to the mitochondria [126]. Further, estrogens have been shown to affect concentrations and localization of anti-apoptotic protein [92,99,126-128], which appear to exert their anti-apoptotic effects through maintenance of mitochondrial membrane potential in the face of stresses [69]. Thus, it is reasonable that the mitochondria are a major site of the neuroprotective effects of estrogens. To assess this possibility, we undertook a series of studies to determine if estrogens affect mitochondrial functions, particularly in the face of stresses that are known to compromise this vital organelle.

6.a. Effects of Estrogens on ATP Production

We assessed the effects of estrogens on the production of ATP under two condition of stress to the mitochondria [103,129,130]. We first determine the effects on ATP production of 3-nitropropionic acid (3NPA), a succinate dehydrogenase inhibitor that uncouples oxidative phosphorylation, and the capacity of estrogens to ameliorate the effect on ATP of this mitochondrial toxin. Second, we induced a compromise in mitochondrial function through the

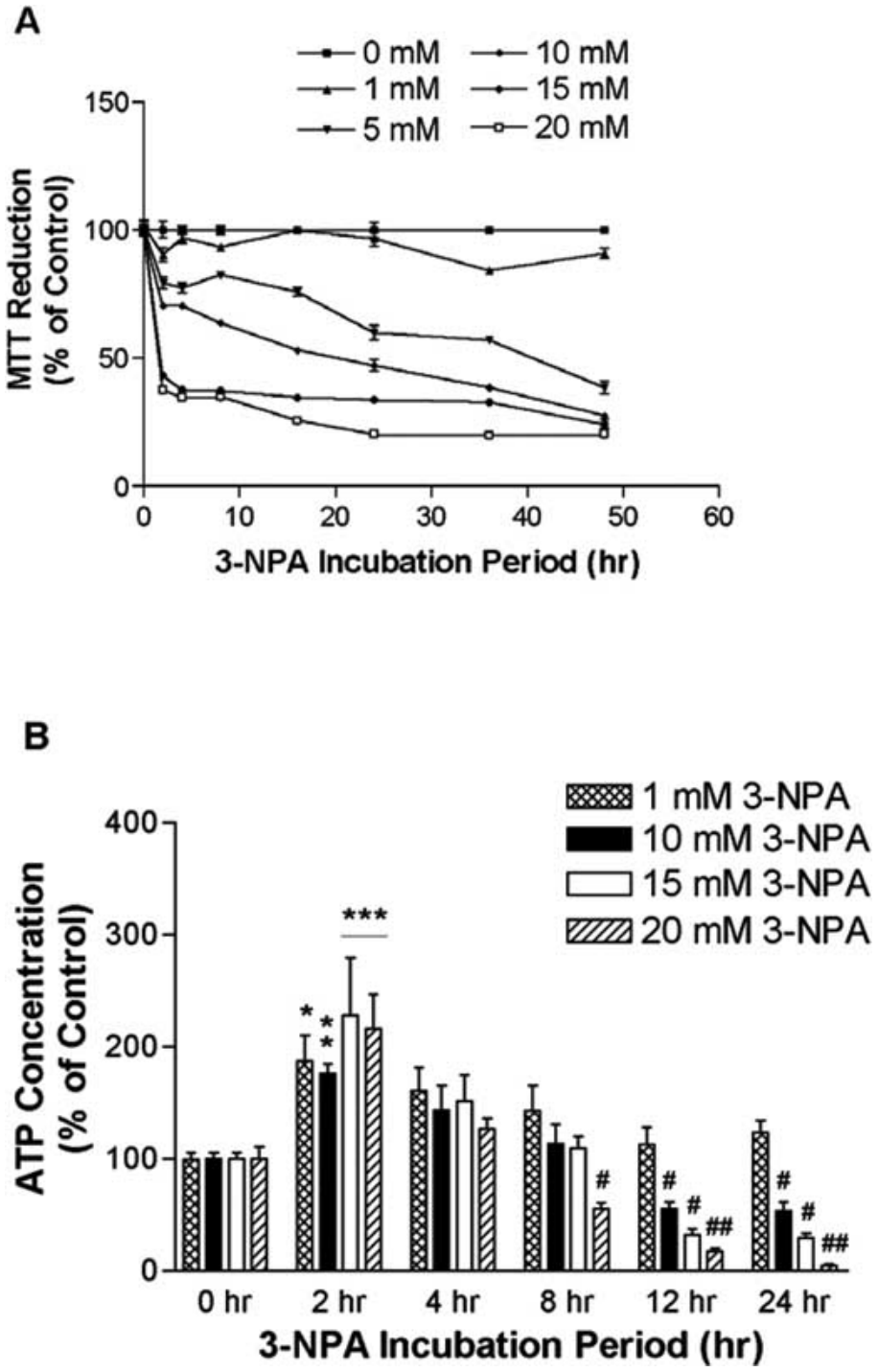

Fig. (2). Effect of 3-nitroproprionic acid (3-NPA) on MTT reduction and cellular ATP levels. (A) Time course of the effects of various doses of 3-NPA on MTT reduction in SK-N-SH cells. Values are expressed as a percentage of control levels and represent the mean ± SEM of determinations made in 8 cultures/group. The control values were the values in untreated cultures and were 0.863 ± 0.053 absorbance units per culture. (B) Time course of the effects of various doses of 3-NPA on ATP concentrations in SK-N-SH cells. Values are expressed as a percentage of control levels and represent the mean ± SEM of determinations made in 3-6 cultures/group. Basal ATP levels in control cultures were 32.6 ± 7 nmol/mg protein. *p<0.05, **p<0.01, ***p<0.001; #p<0.05, # #p<0.01 compared to 0 hr groups.

exogenous administration of the ROS, H_2O_2 and assessed the effects of estrogens on this response.

3NPA effectively suppressed succinate dehydrogenase activity (Fig. **2A**). 3NPA causes a transient increase, followed by a dose- and time-dependent decrease in cellular ATP levels (Fig. **2B**). This indicated that inhibition of complex II by 50% or greater causes a marked and sustained reduction in the ability of the mitochondria to produce ATP. Cultures were pre-treated with 17 β-estradiol (E2) for 6 hours, followed by 3NPA treatment (10mM, a concentration

that reduced ATP by 50% at 12 hours) (Fig. **3**). E2, in the absence of 3NPA, had no effect on ATP level, suggesting that under basal, non-stress conditions, E2 has little effect on cellular energy production *in vitro*. In contrast, pre-treatment with E2 dose-dependently attenuated the 3NPA-induced decrease in ATP production (Fig. **3**).

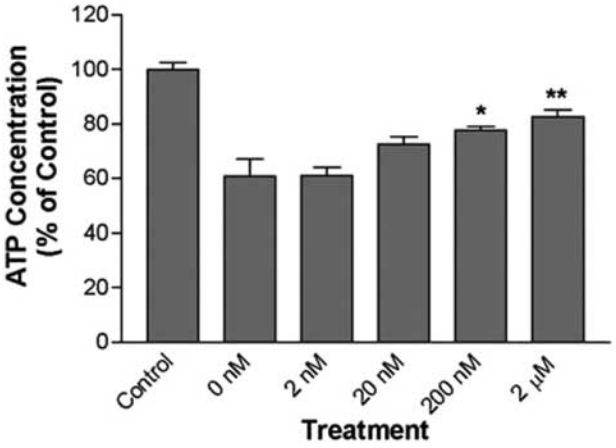

Fig. (3). Effect of 6 hr 17β-E2 pretreatment on ATP levels in SK-N-SH cells at 12 hr of 10 mM 3-NPA treatment. Values are expressed as a percentage of basal levels and represent the mean ± SEM of determinations made for 4-8 cultures/group. Basal ATP levels in control cultures were 46.8 ± 7 nmol/mg protein. *$p<0.05$, **$p<0.01$ compared to the 0 nM 17β-E2 group.

Similarly, administration of H2O2 to neuronal cells in culture caused a dose-and time-dependent decline in ATP production [130,131] (data not shown), demonstrating that exogenous ROS severely compromise mitochondrial oxidative phosphorylation. Pre-treatment with E2 ameliorated the H_2O_2-induced decline in cellular ATP [130,131] (data not shown). This ability of E2 to prevent pro-oxidant-induced declines in cellular ATP appears to be a general property of E2, as we have demonstrated a similar effect of E2 against H_2O_2-induced decline in cellular ATP in a non-neuronal, human lens cell type [129] (Fig. **4**). Collectively, these data indicate that while estrogens have little effect on mitochondrial ATP production under basal condition, it is a potent stabilizer of ATP production during pro-oxidant stress. This ability to maintain oxidative phosphorylation in the face of compromising stresses may explain the ability of estrogens to potently protect neurons from a variety of insults, both *in vitro* and *in vivo*.

6.b. Effects of Estrogens on ROS

While the mechanism by which E2 is able to maintain oxidative phosphorylation even in the face of pro-oxidant stress is not yet clearly elucidated, there are several potential mechanisms. First, E2 could prevent production of mitochondria-damaging ROS caused by the insult. Estrogens are known to exert anti-oxidant effects [97,132-137], but are comparatively poor scavengers of ROS in aqueous media. In both neuronal [130,131] and non-neuronal cells [129] exposed to H_2O_2, E2 is ineffective in reducing cellular ROS levels as measured by general ROS dyes. In contrast, E2 was very effective in preventing the production of H_2O_2 as well as peroxynitrite induced by 3NPA treatment (Fig. **5**). This effect of E2 could be primary in its ability to stabilize ATP production, or could be secondary to other effects of E2.

The observation that estrogens are potent in preventing ROS production, but not in scavenging ROS once formed, lead us to evaluate how estrogens may serve this important role, particularly with regard to inhibition of lipid peroxidation. Two separate pieces of evidence suggested to us a potential mechanism. First, in neuroprotection assays *in vitro*, we demonstrated that estrogens interact synergistically with the high abundant aqueous soluble anti-oxidant, glutathione [89,138]. Second, estrogens are highly lipid soluble (the logarithm of the octanol/water partition coefficient, log P, is 3.35) and largely reside in the

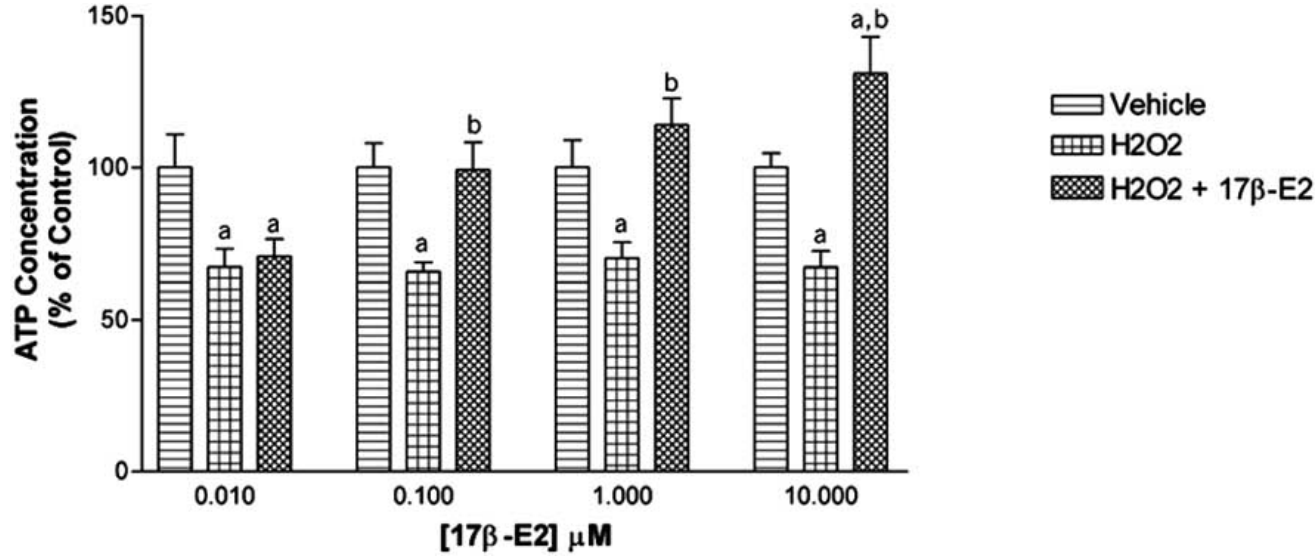

Fig. (4). Effect of 2h 17β-E_2 pretreatment on ATP levels in HLE-B3 cells treated for 90 min with 100μM H_2O_2. The Vehicle group was pretreated with the equal amount of vehicle. Values are expressed as a percentage of normal levels and represent the mean ± SEM of determinations made in 6-8 cultures/group. **a** indicates $p < 0.05$ vs. vehicle group and **b** indicates $p < 0.05$ vs. H2O2 treat only group.

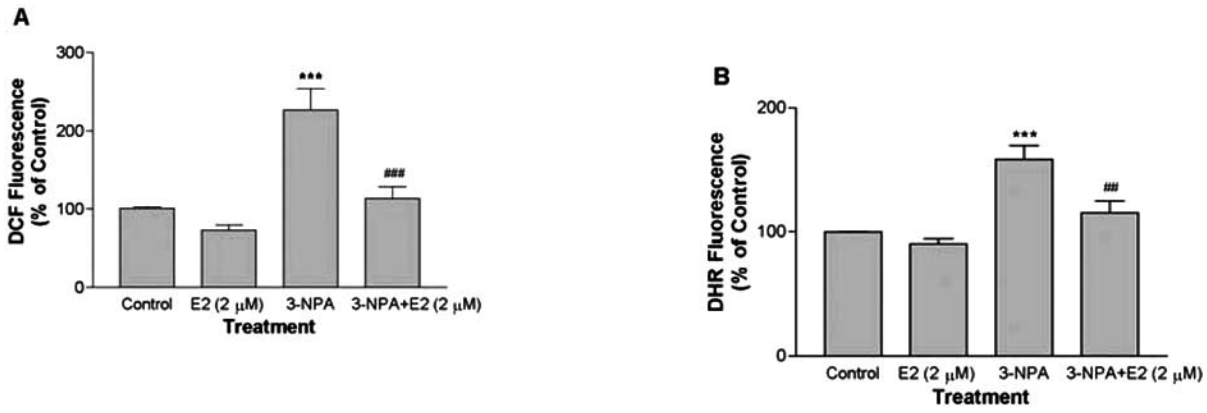

Fig. (5). Effect of pretreatment with 17β-E2 for 6 hr on (A) hydrogen peroxide and (B) peroxynitrite levels after exposure to 2 hr of 3-NPA. Cultures were exposed to the indicated treatments. Values are expressed as a percentage of controls and represent the mean ± SEM for 4-6 cultures/group. The control values are 223 ± 3 and 325 ± 4 fluorescence units for DCF fluorescence and DHR fluorescence, respectively. # # pversus 3-NPA group. ###< 0.05 p<0.01 compared with the 3-NPA group; ***p<0.001 compared with control group or E2 alone group.

membrane component of cells [139] where they are ideally suited to affect oxidation of unsaturated bonds in phospholipids. Indeed, estrogens appear to intercalate into the membrane with their phenolic A ring situated near the site of lipid peroxidation (Fig. **6**).

We envisioned that estrogens may prevent lipid peroxidation by sacrificing itself to oxidation in a manner that could be redox-cycled back to the parent estrogen, using a plentiful and regenerable source of cellular reducing potential, such as glutathione or NADPH. We discovered that estrogens were converted under oxidizing conditions to a quinol product that was, in turn, enzymatically reduced back to the parent estrogen in the presence of NAD(P)H as a co-factor [140,141]. This estrogen redox cycle (Fig. **7**) is operative in brain [140] where it serves, together with the "classical" antioxidant mechanism [142] for phenolic compounds indicated by the top arrows of (Fig. **7**), as a defense mechanism against ROS.

6.c. Effects of Estrogens on Ca^{2+} Loading of the Mitochondrial

Estrogens could affect mitochondrial function by directly or indirectly influencing mitochondrial loading with Ca^{2+}. Brinton's laboratory [128,143] has demonstrated that with mild glutamate stimulation, estrogens enhance Ca^{2+} flux

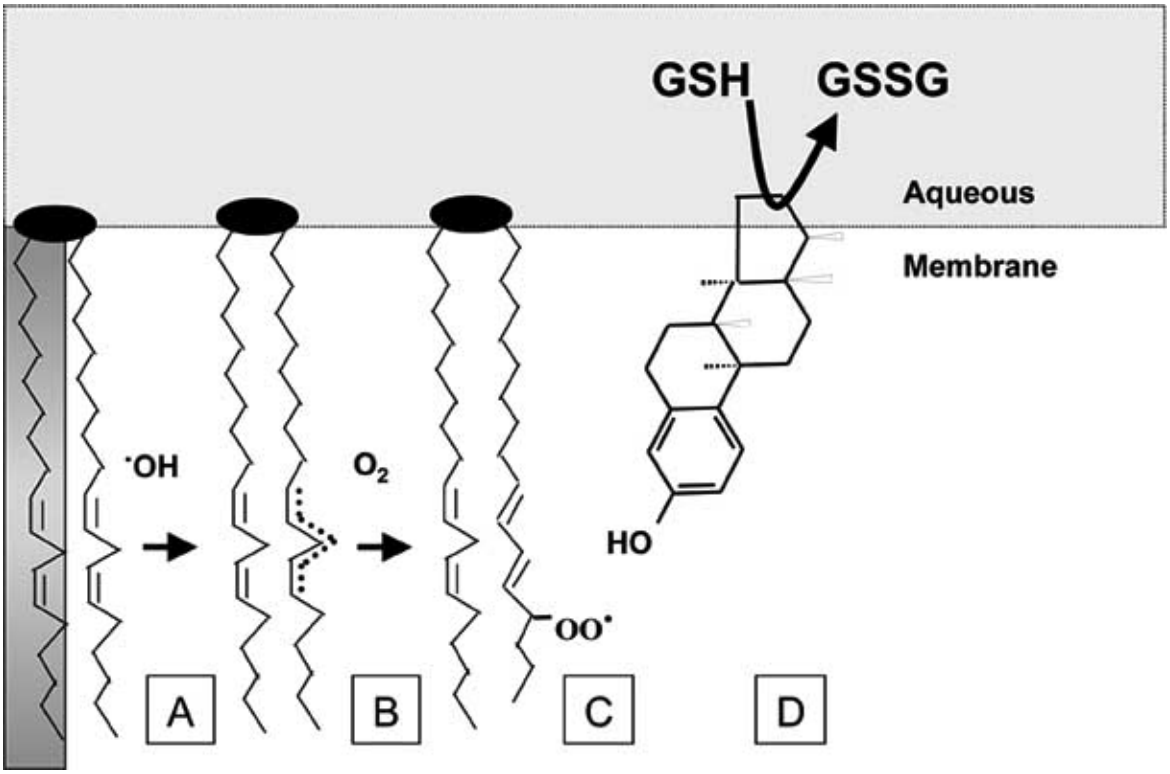

Fig. (6). Schematic illustration of the continuous action of estrogens as neuroprotectants by hydroxyl-radical capture.

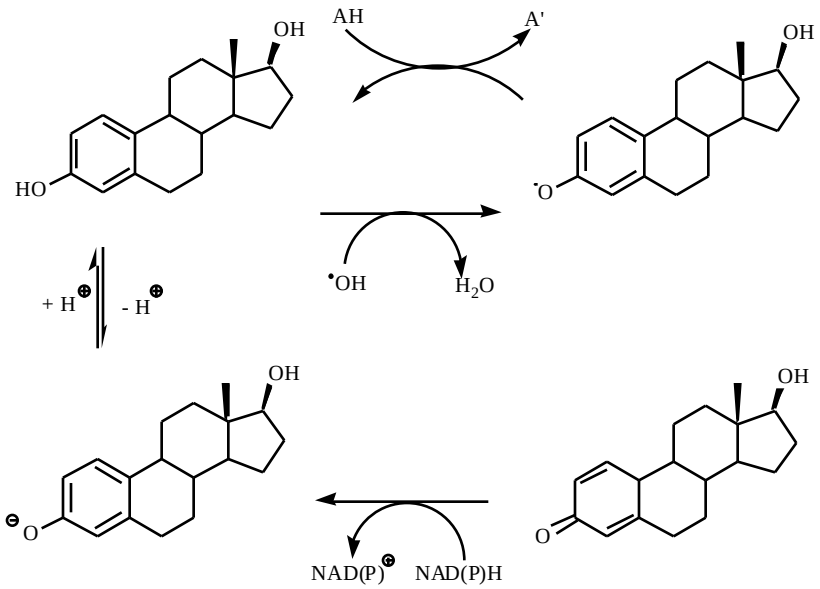

Fig. (7). Schematic representation of the estrogen redox cycle. The direct oxyradical-scavenging activity of phenolic A-ring estrogen involves a radical-exchange reaction that produces the corresponding phenoxyl-radical. Estrogen may then be regenerated by a reaction with ascorbic acid (AH, Vitamin C) [151] or through GSH-dependent free-radical reductase (152). Alternatively, the phenoxyl-radical may recombine with $^{\cdot}OH$ to give a *para*-quinol that is converted back to estrogen by enzymatic reduction involving NAD(P)H as a co-factor [140, 141].

into cells, an effect that may be involved in estrogen's ability to increase memory function through this NMDA mediate mechanism [144,145]. At high levels of excitotoxic stimulation however, estrogens prevented both cytosolic and mitochondrial influx of Ca^{2+} [128,143], presumable providing a protection from Ca^{2+} influx. Comparable effects on mitochondrial stability and function have been reported by Morin *et al*. [146] who studied the ability of 17 α-estradiol, an isomer of 17 β-estradiol that is equipotent as a cytoprotectant yet at least 200-fold less active than as a hormone [147] to maintain respiratory coupling after imposed ischemia-reoxygenation.

We assessed the effects of mitochondrial toxins on cytosolic and mitochondrial loading of Ca^{2+} and determined the dose-dependence of estrogen protection from these effects [103]. 3-NPA caused a rapid and profound increase in cytosolic Ca^{2+} concentrations (Fig. **8A**). Pre-treatment with

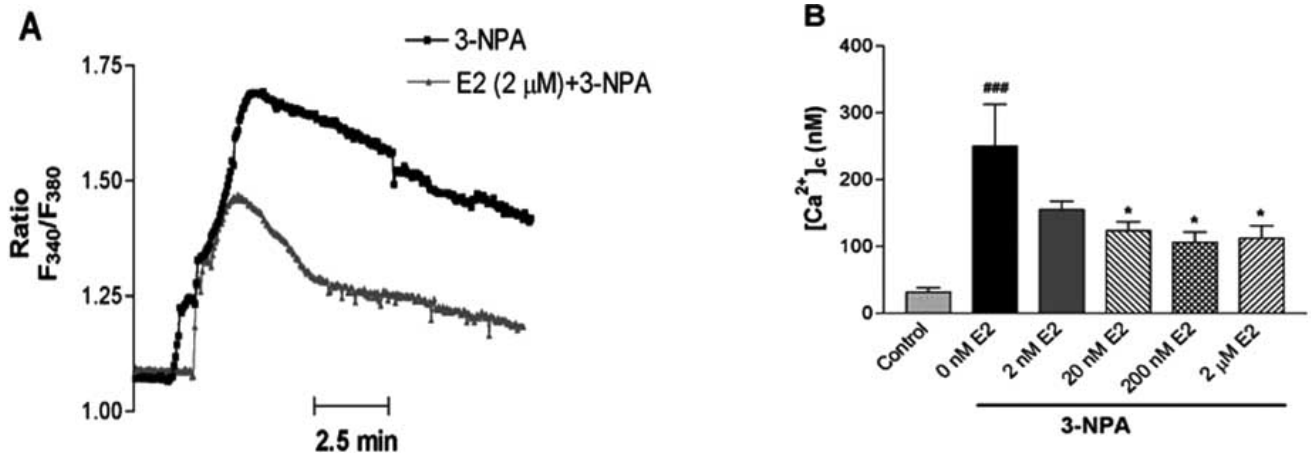

Fig. (8). Effect of 17β-estradiol on 3-NPA-induced changes in peak $[Ca^{2+}]c$ in SK-N-SH cells. (A) Cells were exposed to17β-estradiol for 4 hr. **(A)** The real time changes of fura-2 fluorescence intensities were recorded using a MetaFluor system with a 40 X objective. The excitation wavelength was alternated between 340 and 380 nm, and emission fluorescence was recorded at 510 nm. The fluorescence ratio was calculated as F_{340}/F_{380}. The system was calibrated using solutions containing either no Ca^{2+} or a saturating level of Ca^{2+}. The equivalent in Ca^{2+} concentration was calculated using the formula: $[Ca^{2+}]c = Kd [(R – Rmin) / (Rmax – R)] (Fmin / Fmax)$. **(B)** Dose-dependent effect of 17β-estradiol on 3-NPA-induced changes in $[Ca^{2+}]c$. Data bars represent the mean ± SEM of measurement in 10 to 20 cells from 6-8 pictures. $^{###}$, $p<0.001$ compared to the control groups. *, $p<0.05$, 3-NPA-induced increase in $[Ca^{2+}]c$ in the presence of 17β-estradiol is significantly different from vehicle controls (0 nM E2).

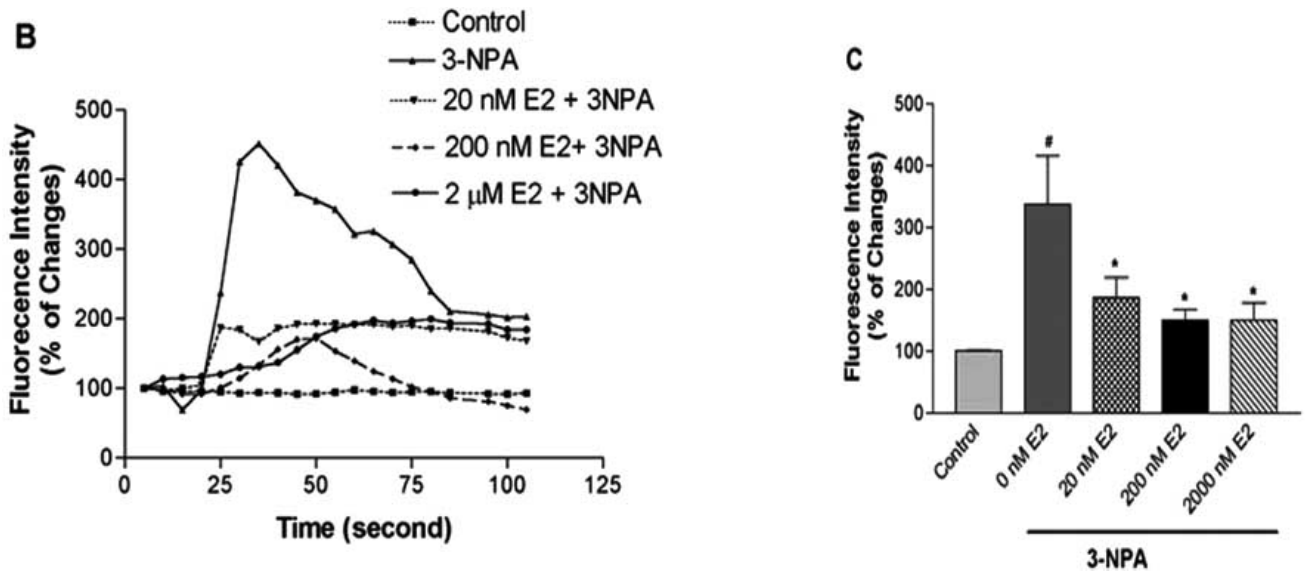

Fig. (9). Effects of 17β-estradiol on 3-NPA-induced increase in mitochondrial calcium levels. SK-N-SH cells were exposed to17β-estradiol at doses ranging from 20 nM to 2 μM for 4 hrs. After incubation with 2 μM fluorescence dye rhod-2 AM for 45 mins, cells were washed with physiological buffer. Cells were then treated with 10 mM 3-NPA. The calcium variations were monitored by fluorescence confocal microscopy, and a time series of images was recorded before and after cell stimulation. Time series of 20 confocal images were recorded for each experiment, each series containing images recorded at time intervals of 5 sec. A 488-nm laser was used for the dye excitation, and a 515-nm long-pass filter was used for emission. **A.** Rhod-2 fluorescence (stained mitochondria-omitted for space considerations). **B.** Rhod-2 fluorescence intensities at various time points before and during 3-NPA stimulation. **C.** Data represent the mean ± SEM of changes in rhod-2 fluorescence intensities upon stimulation in four independent experiments. Data were analyzed using ANOVA following by Tukey's post hoc test. # $p<0.05$ compared to the vehicle group; * $p<0.05$ compared to the 3-NPA group.

E2 dose-dependently reduced the influx of Ca^{2+} into the cytosol with a 50% reduction at 20 nM E2 (Fig. **8B**). Similarly, 3NPA caused a rapid and 3-fold influx of Ca^{2+} into the mitochondrial (Fig. **9A**), as effect that was dose-dependently reduced by E2 (Fig. **9B**). We have observed essentially the same protection of cytosolic and mitochondrial Ca^{2+} levels by E2 when H_2O_2 was used as a pro-oxidant mitochondrial toxin [130,131].

In view of the observation that sustained increases in mitochondrial Ca^{2+} impair oxidative phosphorylation and cause an increase in ROS, our observations suggest that the Ca^{2+} modulating effects of estrogens may serve to protect ATP production and thereby neuronal viability.

6.d. Effects of Estrogens on Δψm

Mitochondrial membrane potential (Δψm) collapse is a critical event in the life-death decision of neurons [1,44,45,54,148]. We used two methods to determine the effects of mitochondrial toxins and of E2 on Δψm in neuronal cultures. First, analysis of rhodamine 123, a mitochondrial specific dye, demonstrated that 3NPA caused mitochondrial depolarization (Fig. **10**) and this effect of the mitochondrial toxic was antagonized by E2 pre-treatment (Fig. **10**). Similarly, using a FRET assay to measure Δψm [149], we observed that treatment with either E2 or its diasteriomer, 17 α-estradiol (17 α-E2) increased the Ca^{2+} concentration required to cause Δψm collapse (Fig. **11**). These effects of E2 and 17 α-E2 are similar to that which we have previously reported [150]. This increase in the ED_{50} of Ca^{2+} could be due to a partial resistance of all mitochondria to Ca^{2+} or complete resistance of a subpopulation of mitochondria in the presence of estrogens. Collectively, these data indicate that estrogens protect mitochondria by preventing mitochondrial membrane potential collapse. This then, could explain the described ability of estrogens to prevent the release from mitochondria of apoptotic factors [2] that is dependent on Δψm collapse.

6.e. Relationship Between Estrogen Neuroprotection and Δψm Collapse

A critical test of the role played by mitochondrial actions of estrogens in their ability to neuroprotect is to define a correlation between the potency of compounds in assays of neuroprotection and mitoprotection. It is presumed that if the neuroprotective effects of estrogens are medicated by a mitochondrial action, the two parameters will correlate strongly. We tested the relationship between the neuroprotective activity of estrogens and their ability to protect from Δψm collapse induced by Ca^{2+} loading of HT-22 cells in culture. Fourteen estrogen analogues were selected for comparison that ranged in neuroprotective potency (ED_{50}) from 20 nM to 8.6 μM (essential ineffective in cytoprotection assays) were selected for comparison. Cells exposed to these compounds were subjected to a Ca^{2+} loading dose-response following permeabilization with

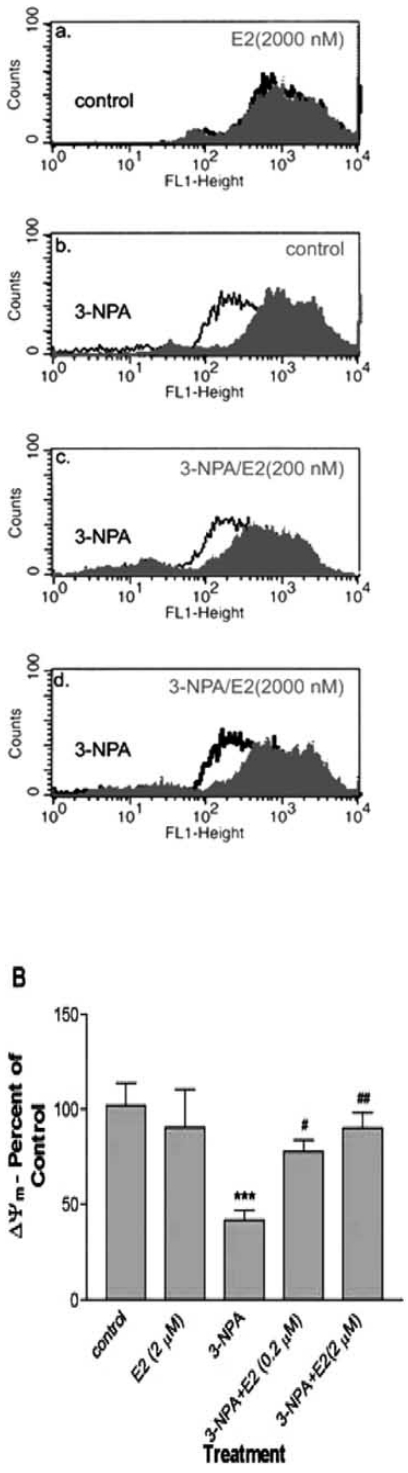

Fig. (10). Effect of 6 hr pretreatment with 17β-E2 on 3-NPA induced changes in mitochondrial membrane potentials. (A) Analysis of rhodamine 123 fluorescence in SK-N-SH cells by FACScan. Cells were positively stained with rhodamine 123. Increasing fluorescence intensities were plotted on the x axis in log fluorescence units versus cell number on the y axis. The FL-1 "high" population is displayed in gray on the histograms and the FL-1 "low" population is represented by the black overlay. Panel a. Cells treated with 2 μM 17β-E2 (gray) and control cells (black). Panel b. Control cells (gray) and cells treated with 10 mM 3-NPA for 12 hr (black). Panel c. Cells exposed to 10 mM 3-NPA for 12 hr with 6 hr 17β-E2 (0.2 μM) pretreatment (gray) and cells only exposed to 10 mM 3-NPA for 12 hr (black). Panel d. Cells exposed to 10 mM 3-NPA for 12 hr with 6 hr 17β-E2 (2 μM) pretreatment (gray) and cells only exposed to 10 mM 3-NPA for 12 hr (black). (B) Quantitative summary of rhodamine 123 fluorescence studies, comparing changes in relative fluorescence intensities under indicated treatments. Values are expressed as a percentage of control levels and represent the mean ± SEM for 4-8 cultures/group. The control value was 159 ± 7 fluorescence units. #p<0.05 compared to the 3-NPA group. # #p<0.01 compared to the 3-NPA group. ***p<0.001 compared to the control or 17 β-E2 group.

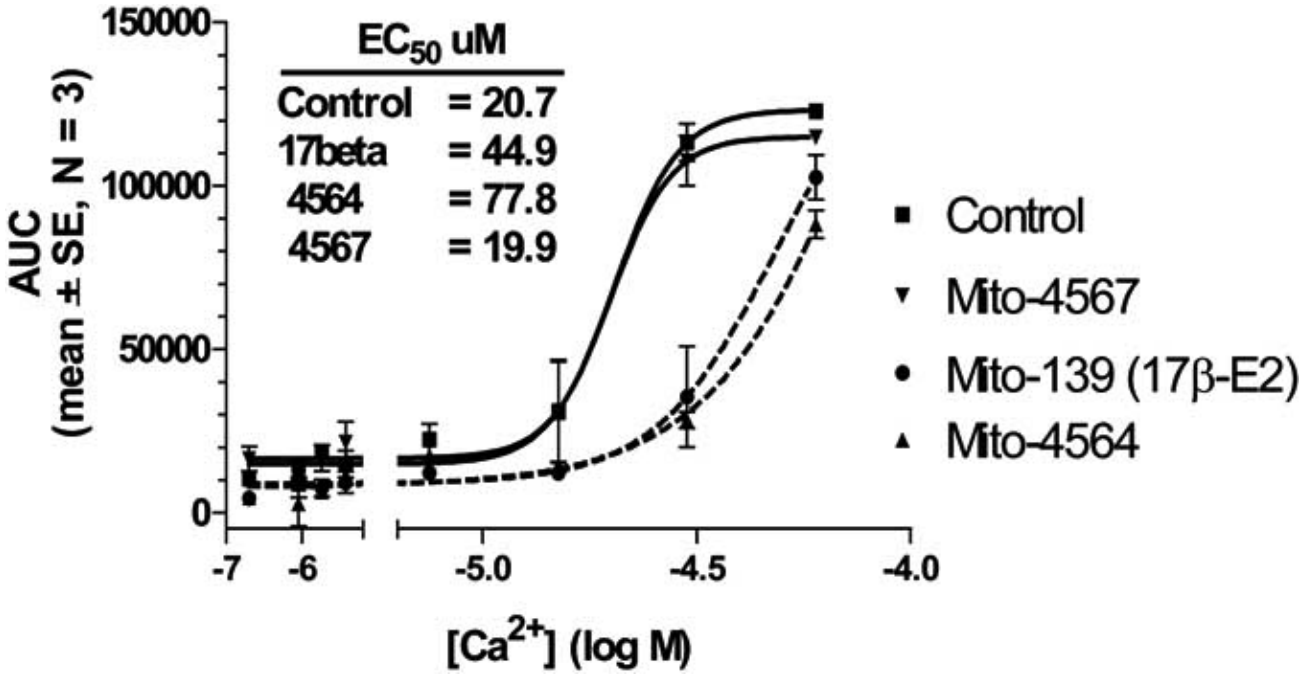

Fig. (11). Effects of 17 β-E2, a neuroprotective estrogen analogue (Mito-4564), and an inactive estrogen analogue (Mito-4567) on mitochondrial membrane potential in response to Ca^{2+} loading. Human SHSY-5Y neuroblastoma cells were cultured in DMEM supplemented with 10% fetal calf serum. Cells were plated 24 hr prior to Ca^{2+} exposure. Mitochondrial membrane potential was measured using FRET assay [146] with a direct Ca^{2+} addition to induce mitochondrial collapse. Compounds were present @ 1 uM for 5 min prior to Ca^{2+} addition. Depicted is the mean for N=3 wells per group, with EC_{50} for the non-linear regressions.

digitonin (0.008%, final concentration for 5 min). Using ten compounds that showed cytoprotective activity, the correlation between ED_{50} values for neuroprotection and the ED_{50} values for Ca^{2+}-induced Δψm collapse was highly significant (r^2 = 0.73, Spearman r = -0.9387, p<0.0001) (Fig. **12**). It is worth noting that 4 compounds that failed to show cytoporotective activity also failed to stabilize mitochondria. However, these compounds were not included in the above correlation analysis because the absence of cytoprotection activity artifactually skewed the regression; when the 4 ineffective compounds were also included in the analysis, the linear regression r^2 drops to 0.28 (P = 0.0503), while the correlation remains highly significant (Spearman r = -0.8543, P < 0.0001; n=14).

The significant correlation between neuroprotection and mitochondrial protection is particularly impressive in view of the fact that the ED_{50} values for the two parameters were derived from data generated in two separate laboratories. This strong correlation suggests that the effects of these estrogen-like compounds on mitochondrial protection can explain most of the neuroprotective activity of estrogens in this cell type.

7. CONCLUSIONS

Mitochondria failure is central to most neuronal death pathways induced by a wide variety of insults, and depending upon the severity of the insult, can yield necrosis or apoptosis. Estrogens have multiple effects on mitochondrial function that are expressed under stress. They preserve ATP production, prevent production of ROS, resist cellular and mitochondrial Ca^{2+} loading and preserve mitochondrial membrane potential during insults. All of these effects are important in maintaining the viability of mitochondria during insults. In brief, estrogens appear to render mitochondria resistant to insults that result in their initiation of necrotic as well as apoptotic cell death pathways.

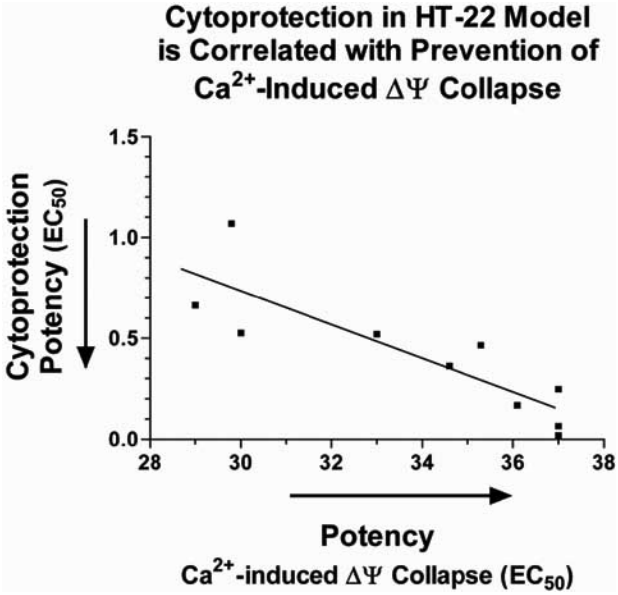

Fig. (12). Correlation between neuroprotective activity and protection from Ca^{2+}-induced mitochondrial membrane potential collapse in HT-22 cells. Ten compounds were assessed for protection against glutamate toxicity and protection from mitochondrial membrane potential collapse. The Spearman r = -0.9387, r2 = 0.73 and p< 0.0016.

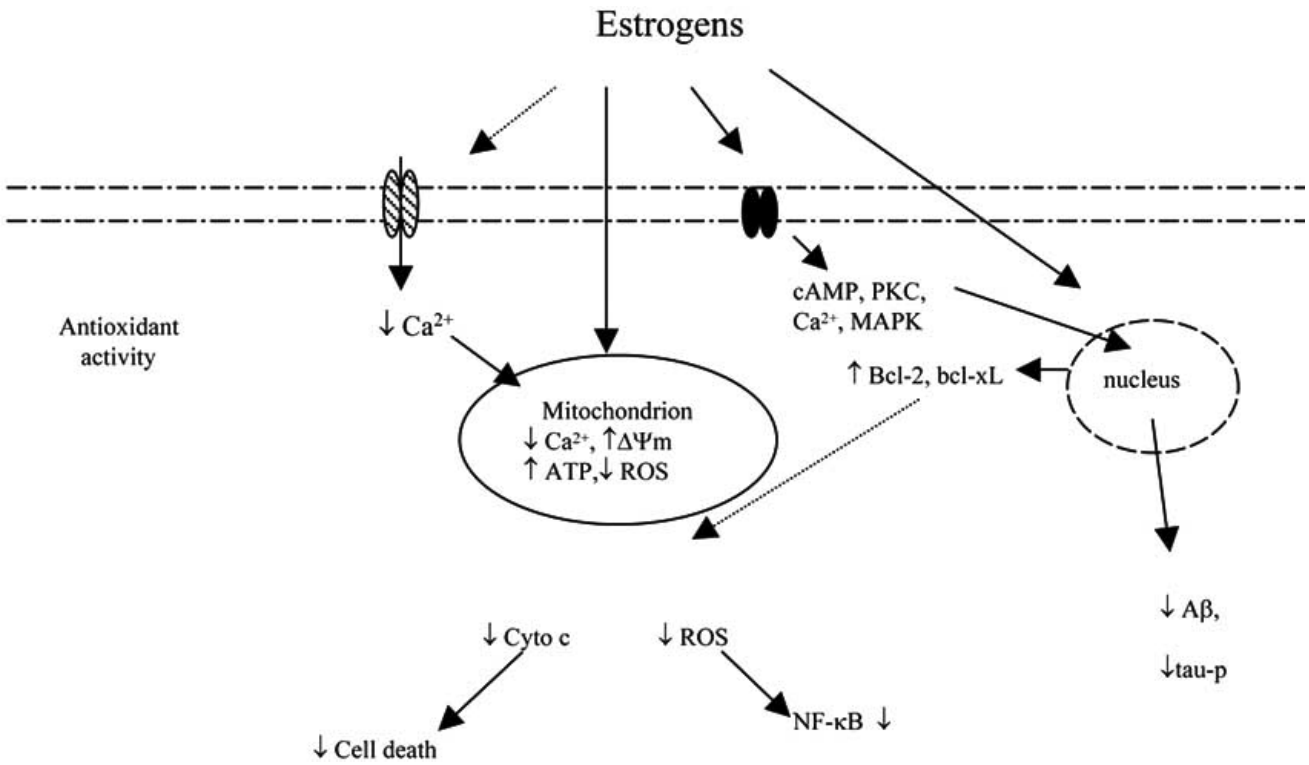

Fig. (13). Schematic diagram of pathways involved in protection of mitochondrial function by estrogen. Dash line represents inhibition of activity. Solid line represents activation. Abbreviations used: Ca^{2+}, calcium; cAMP, cyclic adenosine monophosphate; PKC, protein kinase C; MAPK, mitogen activated protein kinase;Δψm, mitochondrial membrane potential; ATP, adenosine triphosphate; ROS, reactive oxygen species; Cyto C, cytochrome C; NFκB, nuclear factor-κ-B; A,β-amyloid peptide; tau-p, hyperphosphorylated tau protein.

The molecular target of this important effect of estrogens is not yet known, but could be in the mitochondria or in other cellular compartments that communicate with mitochondria (Fig. **13**). A determination of the molecular targets of estrogen action on mitochondrial function is a critical issue that must be resolved to understand estrogen neuroprotection as well as to optimize drugs for the treatment of neurodegenerative diseases. The most parsimonious mechanistic model points to membrane stabilization via redox cycling with glutathione, and this model has already yielded substantial improvements in potency of the estrogen analogs. That such improvements are a worthy goal is underscored by the observation that the mitochondrial functions improved by estrogens are the very functions that are compromised in a variety of neurodegenerative disease and more acutely compromising conditions, like cerebral stroke and brain trauma, thereby providing a novel avenue of therapeutic intervention.

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