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Free Radicals in Neurodegenerative Diseases: Modulation by Palladium α-Lipoic Acid Complex

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1. Introduction

Our research is based on the need for modern medicine to develop a safe and nontoxic product with a wide spectrum of uses. We strongly believe that one of the best ways to achieve this is to have a product that participates actively in most of the roles played by the mitochondria for optimal cellular function. Mitochondria are ubiquitous, and taking care of mitochondria is similar to taking care of all the parts leading to greater achievements than the sum of the parts [Krishnan et. al., 2011].

Oxidative stress is caused by the chemical imbalance between reactive oxygen species (ROS) production and their breakdown by antioxidants. Over-abundance of ROS has been found during neuronal development, as well as in numerous neuropathological conditions. A predominant feature of neuronal injury is the onset of oxidative stress.

Oxidative stress and mitochondrial dysfunction have been closely associated in many sub-cellular, cellular, animal, and human studies of both acute brain injury such as ischemia and stroke and neurodegenerative processes such as Parkinson's, Alzheimer's and Huntington's. While the oxidative stress occurs chronically in Alzheimer's disease, it is more acute in ischemic reperfusion injury. The consequences of mitochondrial dysfunction include DNA and protein damage, lipid peroxidation, disruption of the mitochondrial permeability transition, Ca^{2+} homeostasis, and triggering apoptosis. It is essential to have a healthy mitochondria contributing substantially to the physical, mental, and emotional elements needed to support the well being of patients suffering from brain injury or neurodegenerative diseases.

Energy metabolism, calcium regulation, and apoptosis-signaling pathways are the major roles of mitochondria. Energy requirements dictate the number of mitochondria in a cell [Beattie, 2002; Nagley et. al., 2010]. Cardiac and skeletal muscles, the brain, and the liver have the most mitochondria because of their high metabolic activities. These cells are also exposed to the most oxidative stress because the source of free radical production is also the mitochondria. Due to low levels of antioxidants in neurons, they are intrinsically ill-equipped to defend against an increase in oxidative stress. Glial cells including astrocytes play a supplementary role in antioxidant defense of neurons [Higgins et. al., 2010].

Our search for an extremely safe (up to 40 mL/day, 0.037 M aqueous solution) and nontoxic therapeutic agent resulted in the development of a novel redox molecule, "Palladium α-Lipoic Acid Complex" that is active in mitochondrial cellular metabolism and other

functions. The selection of the naturally occurring coenzyme, α-lipoic acid, as our ligand was based on its safety as well as its redox, antioxidant, and fatty acid properties. After selecting the ligand that plays a critical role in biological energy metabolism and numerous other functions, we wanted to tweak the properties of the ligand by complexing it with a metal that is safe and has very high catalytic and electronic properties. After numerous investigations with a variety of metals, the final selection was made to use palladium.

The properties of the resulting palladium α-lipoic acid complex were remarkable in many ways and have been reviewed recently [Krishnan et. al., 2011]. Briefly, this complex enhances the enzymatic activities of Krebs cycle enzymes, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase and mitochondrial respiratory enzymes, complex I, complex II, complex III, and complex IV. These enzymatic activity enhancements by the metal complex were, in general, much greater than that of the ligand, α-lipoic acid. Coupling this increase in the efficiency of the aerobic metabolic cascade with its powerful antioxidant properties, such as scavenging of free radicals, lowering lipid peroxidation, increasing the levels of glutathione, glutathione peroxidase, manganese superoxide dismutase, and catalase, gave us a powerful weapon to combat fatigue associated with numerous mitochondrial abnormalities. The complex also modulates mitochondrial dysfunction, acts as a prophylactic for neuronal protection from transient ischemic attack, repairs DNA damage resulting from radiation, acts as a prophylactic for protection from radiation, and improves the quality of life. The electronic properties corresponding to tunnel diode behavior and the therapeutic ability/potential of this complex may be exploited in its applications for combating brain injury resulting from transient ischemic attack, death of neurons and other progressive loss of structure or function of neurons associated with diseases such as Parkinson's and Alzheimer's.

2. Oxygen (the source of free radicals) and antioxidants

The appearance of oxygen in the atmosphere is associated with a great expansion of the varieties and numbers of higher living forms. Oxygen is the source for the emergence of respiratory metabolism and energy efficiency. It is also the source of free radicals such as hydroxyl and superoxide. Oxygen's imprint on earth's metabolic evolution, the effect of oxygen on biochemical networks, and the evolution of complex life have been reviewed [Falkowski, 2006; Raymond & Segré, 2006].

Oxygen is the most abundant element in the earth's mantle. Its limited solubility in water (48.9 mL of oxygen at 1 atm pressure in 1 liter water at 0°C) makes aquatic life possible [Pauling, 1970]. Ordinary oxygen consists of diatomic molecules with an unusual electronic structure. Instead of having a double bond between the two atoms in molecular oxygen in the ground state, only one shared pair is formed leaving two unshared electrons. This makes the molecule a diradical and paramagnetic. Liquid oxygen exhibits a pale blue color.

A free radical is a highly reactive species with an unpaired electron. It can be a neutral species such as hydroxyl, $HO^{\bullet}$, or a charged negative ion (anion) such as superoxide, $O_2^{\bullet -}$, or a charged positive ion (cation) such as the guanine radical. An unpaired electron is shown as a dot after the symbol (example: $HO^{\bullet}$). Being good oxidizing agents, free radicals can remove an electron from other materials and in that process get reduced with the pairing of the unpaired electron. They often participate in chain reactions producing new free radicals.

Small fluctuations in the steady state concentrations of free radicals play a significant role in intracellular signaling. Uncontrolled increases in the production of these radicals lead to

chain reactions and damage to proteins, polysaccharides, and DNA. The steady state concentrations of $O_2^{\bullet -}$ and H_2O_2 are ~ 10^{-10} M and 5 x 10^{-9} M respectively [Dröge, 2002].
Another beneficial aspect of free radicals is that they participate with leukocytes in phagocytosis, the engulfing and destruction of particulate matter and bacteria. Leukocytes contain the enzymes of the hexose-monophosphate shunt, glycolysis, citric acid cycle, and respiratory enzymes. Phagocytosis requires a lot of energy, which is obtained from glucose by glycolysis and also by the hexose-monophosphate shunt. The role of this shunt is to produce hydrogen peroxide from superoxide free radical, which is used in the phagocytotic process. Thus the free radicals produced in this process are beneficial [Singh, 2006].
Oxidative damage to many biological molecules compromise the viability of cells. The results of this free radical mischief have been assessed [Sies, 1986].
Antioxidants or physiologic reducing agents get oxidized by donating electrons to free radicals. The relative abilities of antioxidants to donate electrons and free radicals to accept electrons are a function of their reduction or redox potentials, measured in volts.
The rate of a reaction cannot be predicted from redox potentials. However the direction of a reaction, decided by the free energy of the reaction, can be predicted from redox potentials. A positive voltage for the net reaction predicts the spontaneity of the reaction. Examples of one-electron and two-electron reduction potentials of reactions of biological interest are easily available [Buettner, 1993; Voet D. & Voet J. G., 1995; Krishnan et.al., 2011]. Positive voltage indicates that vitamin E is spontaneously regenerated by ascorbate or vitamin C.

$$\alpha\text{-Tocopheroxyl}^{\bullet} + \text{Ascorbate monoanion} \longrightarrow \alpha\text{-Tocopherol} + \text{Ascorbate}^{\bullet -},\ E = 218\ \text{mV} \quad (1)$$

The criteria often used to evaluate the antioxidant potential as well as preventive or therapeutic applications of a compound are 1) specificity of free radical quenching, 2) metal chelating ability, 3) interaction with other antioxidants, 4) effects on gene expression, 5) absorption and bioavailability, 6) concentration in tissues, cells, and extracellular fluid, and 7) location (in aqueous or membrane domains or in both) [Packer et. al., 1995].
ROS generation, which increases with increasing stress conditions, is characteristic for all tissues and cells. The interaction of molecular oxygen with biological molecules is not energetically favored because of the unique electronic configuration of molecular oxygen. Molecular oxygen, a diatomic molecule, in its normal or ground state is in its triplet state, $^3O_2$ [$^3\sum^-_g$]. It has two electrons of parallel spins singly occupied in its two π* antibonding molecular orbitals. Most organic molecules cannot react with this spin-forbidden triplet oxygen because of their singlet configurations with antiparallel electron spins. By adding energy, the triplet oxygen can form two types of excited singlet oxygen, $^1O_2$, $^1\sum^+_g$ with the two electrons of opposite spins in two separate molecular orbitals or $^1\Delta_g$ with the two electrons of opposite spins occupying one molecular orbital leaving the other molecular orbital empty. The former is too short lived from a biological point of view. A two electron interaction with molecular oxygen is thus not possible without a spin inversion because it will result in parallel spins in the same orbital, which is spin-forbidden. Thus the preferable interaction is reduction of oxygen by addition of one electron at a time. This process leads to the production of oxygen radicals that can cause cellular damage. When one of the two unpaired electrons is excited and changes its spin, the resulting high energy singlet oxygen with two electrons and opposite spins in the two orbitals is capable of two-electron interactions. The initial step or one electron reduction of oxygen requires energy. The subsequent reduction reactions with appropriate electron donors can proceed spontaneously.

One must wonder at this stage whether nature has given this unique electron configuration for normal molecular oxygen purposefully or not, recognizing not the liability of free radicals but instead their usefulness.

Oxygen undergoes a series of progressive one electron reduction reactions, 2-5. The hydroxyl radical has a very short half life (10^{-9} s) with the highest rate constant with target molecules [Sies, 1993]. It reacts practically at the site of generation. It is one of the strongest oxidizing agents in nature (redox potential of 2310 mV). It is undoubtedly the most dangerous, with its well known involvement in lipid peroxidation of cell membranes and generation of other toxic radicals. The formation of $HO^{\bullet}$ is catalyzed by transition metals in a reduced state. The resulting oxidized metal is reduced back by $O_2^{\bullet -}$ and helps the formation of $HO^{\bullet}$ repeatedly.

$$O_2 + e^- \rightarrow O_2^{\bullet -} \text{ (superoxide anion)} \quad (2)$$

$$O_2^{\bullet -} + e^- + 2H^+ \rightarrow H_2O_2 \text{ (hydrogen peroxide)} \quad (3)$$

$$H_2O_2 + e^- \rightarrow OH^- + HO^{\bullet} \text{ (hydroxyl radical)} \quad (4)$$

$$HO^{\bullet} + e^- + H^+ \rightarrow H_2O \quad (5)$$

Superoxide ion is the precursor of most reactive oxygen species. It can act both as a reducing agent or reductant (for Fe^{3+}) and as an oxidizing agent or oxidant for catecholamines. To minimize production of free radical chain reactions such as reactions (6) and (7), metal ions are sequestered under physiological conditions by proteins.

$$O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (6)$$

$$H_2O_2 + Fe^{2+} + H^+ \rightarrow HO^{\bullet} + Fe^{3+} + H_2O \text{ (classical Fenton reaction)} \quad (7)$$

Adding reactions (6) and (7) gives the Haber-Weiss reaction (8) which is catalyzed by metal ions.

$$H_2O_2 + O_2^{\bullet -} + H^+ \rightarrow HO^{\bullet} + O_2 + H_2O \quad (8)$$

Damage to both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) may result in mutations. Nonspecific binding of Fe^{2+} to DNA may result in the formation of $HO^{\bullet}$ (reaction 7) that attack individual bases and cause strand breaks.

Strategies of antioxidant defense in terms of prevention, intervention, and repair have been elegantly summarized [Sies, 1993]. Cells employ enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase to scavenge free radicals. These enzymes are known as preventive antioxidants because they eliminate the species involved in the initiation of free radical chain reactions. SOD has three isoforms: Copper/Zinc SOD, Manganese SOD, and extracellular SOD. A very high rate of production of ~ 300 nmol superoxide/min/mg protein has been reported for a reaction of cyrtochromeb5 reductase using NADH as an electron donor [Starkov & Wallace, 2006]. The superoxide anion shown in reaction (2) and released by the mitochondria undergoes the dismutation reaction either spontaneously or catalytically(reaction 9) producing hydrogen peroxide and O_2. The role of

the SOD is to increase the rate of the reaction to that of a diffusion controlled process [Turrens, 2003].

$$2H^{+}+O_2^{\bullet -}+O_2^{\bullet -} \xrightarrow{SOD} H_2O_2+O_2 \tag{9}$$

The spontaneous decomposition of superoxide, but not the catalytic decomposition, produces singlet oxygen instead of the normal molecular oxygen [Klotz, 2002]. Singlet oxygen is produced by stimulated neutrophils in vivo. Superoxide produced by NADPH catalysis spontaneously dismutates to H_2O_2 and singlet O_2. Also the catalytic reaction of myeloperoxidase with H_2O_2 and Cl^- produces hypochlorite, which reacts with more H_2O_2 producing singlet O_2, H_2O and Cl^-.

Hydrogen peroxide is removed by GSHPx, at the expense of glutathione or γ-glutamylcysteinylglycine (GSH), and by catalase. Selenium (as selenocysteine) is a cofactor of GSHPx [Beattie, 2002]. GSHPx is located in both the mitochondrial and cytosolic compartments of the cell. Hydrogen peroxide and organic hydroperoxides in the cytosol are also destroyed by GSHPx. Catalase is highest in peroxisomes and it is less in cytosol and mitochondria [Beattie, 2002].

$$2GSH+H_2O_2 \xrightarrow{GSHPx} GSSG+2H_2O \tag{10}$$

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O+O_2 \tag{11}$$

Glutathione or, GSH is a major nonenzymatic antioxidant. The aqueous compartments of cells and their organelles usually contain millimolar levels of GSH. It is the cell's primary preventative antioxidant. It can react with various highly oxidizing species such as $HO^{\bullet}$, $RO^{\bullet}$ or $ROO^{\bullet}$ and produce H_2O, ROH, or ROOH and $GS^{\bullet}$ (glutathiyl radical). Glutathiyl radical can react rapidly with GSH, most efficiently via GS- to make GSSG·-, which is a very strong reducing species. It produces $O_2^{\bullet -}$ and glutathione disulfide, GSSG, by reaction with oxygen.

$$GSSG^{\bullet -}+O_2 \xrightarrow{GSHPx} O_2^{\bullet -}+GSSG \tag{12}$$

SOD and GSH provide an excellent natural combination for cellular antioxidant defense by removing $O_2^{\bullet -}$ and $HO^{\bullet}$ respectively. The intracellular concentration of GSH is about 1 mM while the mitochondrial respiration keeps O_2 about 0 to 10 μM in the cell. Therefore, 99% of GS· formed should react with GSH to make GSSG and $O_2^{\bullet -}$. Thus the importance of SOD is obvious. The normal GSH to GSSG ratio in erythrocytes is 100:1 [Beattie, 2002].

Lipid (probably a polyunsaturated fatty acid) peroxidation is a free radical chain reaction process. Superoxide is a mediator in oxidative chain reactions. It is known to initiate as well as terminate this process. Overproduction of superoxide initiates the chain reaction by mobilizing iron from the tissue protein ferritin [McCord, 1998].

$$O_2^{\bullet -}+\text{Ferritin-}Fe^{3+} \rightarrow O_2+\text{Ferritin}+Fe^{2+} \tag{13}$$

$$Fe^{2+}+\text{lipid-OOH(lipidhydroperoxide)} \rightarrow \text{Lipid-}O^{\bullet}+OH^{-}+Fe^{3+} \tag{14}$$

$$\text{Lipid-}O^{\bullet}+\text{lipid-H} \rightarrow \text{Lipid}^{\bullet}+\text{lipid-OH} \tag{15}$$

$$O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (16)$$

Chain propagation reactions follow.

$$Lipid^{\bullet} + O_2 \rightarrow Lipid\text{-}OO^{\bullet} \quad (17)$$

$$Lipid\text{-}OO^{\bullet} + Lipid\text{-}H \rightarrow Lipid\text{-}OOH + Lipid^{\bullet} \quad (18)$$

If alkoyl (lipid-$O^{\bullet}$) or dioxyl (Lipid-$OO^{\bullet}$) radicals are scavenged by $O_2^{\bullet -}$, the chain reaction would be terminated.

$$lipid\text{-}O^{\bullet} + O_2^{\bullet -} + H^{+} \rightarrow Lipid\text{-}OH + O_2 \quad (19)$$

$$lipid\text{-}OO^{\bullet} + O_2^{\bullet -} + H^{+} \rightarrow Lipid\text{-}OOH + O_2 \quad (20)$$

Superoxide, in moderate concentrations, initiates lipid peroxidation as well as terminates. Over scavenging of $O_2^{\bullet -}$ by over expressed SOD limits the termination process. At intermediate concentrations, the SOD is able to suppress the lipid peroxidation process and $O_2^{\bullet -}$ concentration is sufficient enough to terminate the chain. Thus it has been found that for a certain level of oxidative stress there is an optimum concentration of SOD [McCord, 1998].

The rate constant values for the bis-allylic hydrogen atom abstraction from polyunsaturated lipids and for the addition to the double bond were found to be the same for hydroxyl radical (10^9), alkoxyl radical (10^6) and peroxyl radical (10^2) $M^{-1}s^{-1}$ [Takahashi & Niki, 1998].

Non-enzymatic antioxidants include water soluble vitamin C (ascorbic acid) and lipid soluble vitamin E. Ascorbic acid, or ascorbate anion at biological pH is also a cofactor in several biosynthetic pathways including the enzyme prolylhydroxylase, which modifies the polypeptide collagen precursor to facilitate the formation of collagen fibers.

Due to resonance, the ascorbate radical has a long half life of 1 second. Ascorbate can be oxidized in two successive one-electron steps to ascorbate free radical and dehydroascorbic acid respectively.

$$\text{Ascorbate anion} - e^{-} \longrightarrow \text{Ascorbate free radical} \quad (21)$$

$$\text{Ascorbate free radical} - e^{-} \longrightarrow \text{dehydroascorbic acid} \quad (22)$$

The oxidation of ascorbate to the radical and dehydroascorbic acid can easily be reversed by the enzyme systems that use NADH or NADPH.

$$NADH + 2Ascorbate^{\bullet -} \longrightarrow NAD^{+} + 2\text{Ascorbate monoanion} \left[E = 597\ mV\right] \quad (23)$$

Vitamin E has eight different related homologues, the most abundant being α-tocopherol. The dynamics of this antioxidant, that acts only in lipid domains and quenches lipid peroxyl radicals as well as its numerous other functions, not related to its antioxidant property, such as inhibition of cell proliferation, platelet aggregation, and protein kinase C and 5-lipoxygenase inhibition have been reviewed, raising the possibility that the non-antioxidant mechanisms may contribute to many of the effects previously attributed to antioxidant functions [Ricciarelli et. al., 2002; Niki & Noguchi, 2004].

Vitamin E, and vitamin C, cooperate to protect lipids and lipid structures against peroxidation. Also vitamin C regenerates vitamin E (reaction 1), thereby permitting vitamin E to function again as a free radical chain breaking antioxidant. Other antioxidants such as ubiquinols and GSH also regenerate vitamin E and help to maintain its concentration (< 0.1 nmol/mg membrane) in spite of a very high lipid peroxyl radical generation rate of 1-5 nmol/mg membrane protein per minute [Packer et.al., 1995].

3. Hydrogen peroxide (H_2O_2)

H_2O_2 in its liquid state, is more strongly associated by hydrogen bonding than pure water. The dipole moment of hydrogen peroxide is 2.1 Debye units compared to 1.84 Debye units for water. This should make ion-dipole interactions stronger with H_2O_2 than with H_2O. The relative interactions of Na^+-H_2O and Na^+-H_2O_2 as well as Ca^{2+}-H_2O and Ca^{2+}-H_2O_2 and interactions of these ions with both the solvents depend on their relative concentrations.
A dilute aqueous solution of hydrogen peroxide is more acidic than water [Cotton & Wilkinson, 1972].

$$H_2O_2 = H^+ + HO_2^- \qquad K_{20^\circ C} = 1.5 \times 10^{-12} \tag{24}$$

The following equilibria suggest that H_2O_2 is a strong oxidizing agent in both acidic and basic solutions.

$$H_2O_2 + 2H^+ + 2e^- = 2H_2O \quad E^\circ = 1.77V \tag{25}$$

$$O_2 + 2H^+ + 2e^- = H_2O_2 \quad E^\circ = 0.68V \tag{26}$$

$$HO_2^- + H_2O + 2e^- = 3OH^- \quad E^\circ = 0.87V \tag{27}$$

The enzyme, monoamine oxidase, located in the outer mitochondrial membrane of mammalian tissues, catalyzes the oxidation of biogenic amines and produces H_2O_2. Other pro-oxidant enzymes include nitric oxide synthases, cyclooxygenases, xanthine dehydrogenase, xanthine oxidase, NADPH oxidase, and myeloperoxidase. Rodent heart and brain can produce H_2O_2 at a rate of 0.5-3 nmol/min/mg mitochondrial protein, which corresponds to about 5-20% of their total oxygen consumption [Starkov & Wallace, 2006].
Hydrogen peroxide and superoxide have been implicated as mediators of vascular and functional changes in hypertension [Tabet et. al., 2004]. They increase vascular contraction, stimulate vascular smooth muscle cell growth, and induce inflammatory responses that are characteristic features of small arteries in hypertension.
Hydrogen peroxide plays a dual role [Lázaro, 2007]. Cancer cells produce high amounts of H_2O_2. These increased levels of H_2O_2 result in DNA alterations, cell proliferation, apoptosis resistance, metastasis, angiogenesis, and hypoxia inducible factor 1(HIF-1) activation. Activation of HIF-1 plays crucial roles in apoptosis resistance, invasion/metastasis and angiogenesis. Many human cancers have over expressed HIF-1. On the other hand, hydrogen peroxide also induces apoptosis in cancer cells selectively and the activity of many anticancer drugs is mediated, at least in part by H_2O_2.
Hydrogen peroxide is less reactive than superoxide and is relatively more stable. It crosses the membrane lipid bilayer through aquaporins [Singh, 2006]. With increasing concentrations

of cellular H_2O_2, its function gradually changes from cell signaling, to cell malignant transformation, and to cell death [Lázaro, 2007]. The mystery surrounding the different roles of hydrogen peroxide may be solved, at least partially, by looking at its electronic properties. Our impedance data suggest that the electronic properties (and consequent circuits) of H_2O_2 are dependent on its concentration. Our data also suggest that one has to take a serious look at another important contribution of H_2O_2, the preferential solvation of ions and its biological consequences [Krishnan et.al., 2011] .

The beneficial aspect of H_2O_2 in cell signaling is emerging. Neurons and brain macrophages produce $O_2^{\bullet -}$ in pathological situations and the H_2O_2 produced from $O_2^{\bullet -}$ increases gap junctional communication in astrocytes [Rouach et. al., 2004]. Examples of signaling processes include the over-oxidation of the cysteine in peroxiredoxins from the cysteine sulfenic acid to cysteine sulfinic acid, and the over-oxidation of methionine residues in proteins to methionine sulfoxide [Sies, 1986; Wood et. al., 2003].

Embryonic and fetal growth are facilitated by a certain amount of redox imbalance or oxidative stress [Maiorino & Ursini, 2002; Dennery, 2010]. These investigations detail the level of oxygen and antioxidant status at the first, second and third trimester of pregnancy. Low levels of H_2O_2 and superoxide produced by human sperm are also crucial for the capacitation process that allows the sperm to penetrate the zona pellucida of the ovum. At low, moderate, and highly oxidative states, proliferation, differentiation, and apoptosis or necrosis respectively are favored suggesting the different functions of ROS depending on their concentrations.

4. Reactive nitrogen species

Nitric oxide, $NO^{\cdot}$, is a neutral free radical with a half life of the order of seconds [Beckman & Koppenol, 1996; Blokhina & Fagerstedt, 2006]. Three types of nitric oxide synthases have been described in mammalian cells: neuronal (nNOS), endothelial (eNOS) and an inducible (immunological) (iNOS). The first two are under the control of Ca^{2+}-calmodulin. The enzyme catalyzes oxygen dependent conversion of L-arginine to citrulline:

$$\text{L}-\text{arginine} + \text{NADPH} + O_2 \xrightarrow{\text{NOS}} \text{citrulline} + NO^{\cdot} + NADP^{+} \tag{28}$$

Mitochondrial NOS distinct from the ones given above has also been reported [Elfering et. al., 2002]. $NO^{\bullet}$ can be converted to other reactive nitrogen species such as nitrosonium cation (NO^{+}), nitroxyl anion (NO^{-}) or peroxynitrite, $ONOO^{-}$ with distinct chemical reactivity and physical properties. Some of the physiological effects may be mediated through the intermediate formation of S-nitroso-cysteine or S-nitroso-glutathione [Dröge, 2002].

ROS are produced at complex I and complex III during respiration. Superoxide ion produced at the mitochondria reacts with $NO^{\bullet}$ to produce peroxynitrite. The rate of this reaction is controlled by the rate of diffusion of the two reactants.

$$NO^{\bullet} + O_2^{\bullet -} \xrightarrow{\text{NOS}} ONOO^{-} \tag{29}$$

The reaction rate for the formation of $ONOO^{-}$ is $6.7 \times 10^9\ M^{-1}\ s^{-1}$. This is ~ 6 times faster than the scavenging of superoxide by copper, zinc superoxide dismutase. Inducible nitric oxide synthase, when expressed, can make substantial amounts of nitric oxide and this will out-

compete SOD for $O_2^{\bullet -}$. In aerobic metabolism about 1-5% of oxygen is reduced to superoxide. However its intracellular concentration is maintained at ~ 4-10 µM by superoxide dismutase. Copper, zinc superoxide dismutase is ~ 0.5% of total soluble proteins in brain.
Physiological levels of $NO^{\bullet}$ binds to cytochrome c oxidase leading to a competitive and reversible inhibition of mitochondrial respiration [Radi et. al., 2002]. Large levels of $NO^{\bullet}$ in mitochondria promote formation of more $O_2^{\bullet -}$ from complex I and the consequent formation of more $ONOO^-$.
The half-life of $ONOO^-$ is 0.05 – 1 s [Sies, 1993]. Mitochondrial scavenging systems for $ONOO^-$ and $ONOO^-$-derived radicals such as carbonate ($CO_3^{\bullet -}$) and nitrogen dioxide radicals ($NO_2^{\bullet}$) are cytochrome c oxidase, GSH and ubiquinol [Radi et. al., 2002]. Superoxide and $ONOO^-$ radicals significantly affect the mitochondrial integrity. Peroxynitrite is a very powerful oxidizing and nitrating agent. It reacts with tyrosine in proteins and produces nitrotyrosines. Nitration of the structural proteins, neurofilaments and actin disrupts the filament assembly leading to major pathological consequences in myocardial ischemia, distressed lung, and amyotropic lateral sclerosis.
The steady state concentrations of $NO^{\bullet}$ and $ONOO^-$ in liver are ~ 36 nM and 2.2 nM respectively based on the assumption of 20 µM intramitochondrial O_2 concentration. But there are claims that O_2 concentration is only 3 µM and not 20 µM [Turrens, 2003]. If this is true the steady state concentrations will be much lower. After cerebral ischemia, NO concentration increases 10-100-fold in a few minutes to 2-4 µM[Beckman & Koppenol, 1996].
Nitric oxide can penetrate the lipid bilayer and diffuse rapidly and isotropically through most tissues without any significant reaction or consumption. The rapid diffusion of nitric oxide between cells allows it to modulate, 1) synaptic plasticity in neurons, 2) the oscillatory behavior of neuronal networks, 3) blood flow, and 4) thrombosis [Beckman & Koppenol, 1996]. Since it reacts with oxy hemoglobin and is destroyed, it cannot be transported through the vasculature.
Nitric oxide diffuses and concentrates in the hydrophobic core of low density lipoprotein (LDL) and inhibits its oxidation [Rubbo et. al., 2002]. On the other hand peroxynitrite is involved in LDL oxidation. Since vascular cells are rich sources of superoxide, peroxynitrite formation is also facilitated. Thus the development of atherosclerosis may be intimately connected to the interactions of these two nitrogen species with LDL.
Both peroxynitrite and singlet oxygen are involved in activating mitogen-activated protein (MAP) kinases that respond to extracellular stimuli such as mitogen, osmotic stress, and proinflammatory cytokines and regulate various activities such as gene expression, mitosis, differentiation, proliferative cell survival, and apoptosis [Klotz, 2002].

5. Mitochondria

Mitochondria has unique roles; production of adenosine triphosphate (ATP) by cellular respiration, production of ROS, the distribution/redistribution of Ca^{2+} pools within cells, and control of apoptosis or programmed cell death.
In the absence of mitochondria, or with mitochondrial dysfunction, ATP is produced by an alternative pathway, anaerobic glycolysis. However, this conversion of glucose to pyruvate is not efficient and produces only 2 molecules of ATP compared to 36 molecules of ATP produced by normal glucose oxidation. The pyruvate and the fatty acids are transported into the mitochondrial matrix. There they are broken down into the acetyl group on acetyl coenzyme A (acetyl-CoA or acetyl-SCoA) and then fed into the Krebs cycle.

The ATP has a half-life of seconds to minutes depending on the cell where it is being continuously hydrolyzed and regenerated. Our average normal consumption and regeneration rate of ATP is ~3mol (1.5 kg) h^{-1}. During strenuous activity this rate increases by an order of magnitude [Voet D. & Voet J. G., 1995].

Oxygen deprivation rapidly deteriorates brain cells because ATP is available for only a few seconds [Voet D. & Voet J. G., 1995]. In muscles and nerve cells, ATP has high turnover rates and phosphocreatine acts as its reservoir.

$$\text{ATP} + \text{Creatine} = \text{Phosphocreatine} + \text{ADP} \tag{30}$$

This is an energy consuming reaction under standard conditions and is close to equilibrium under normal intracellular concentrations. At resting state, high ATP shifts the equilibrium to the right. High metabolic activity shifts the equilibrium to the left due to low ATP.

The breakdown of carbohydrates, lipids, and proteins produce the acetyl group of the common intermediate, acetyl-CoA. A series of consecutive enzymatic reactions of Krebs cycle, the electron transport chain (ETC), and oxidative phosphorylation then converts acetyl-CoA into CO_2 and H_2O. The net result is the transfer of electrons from the oxidative substrates to molecular oxygen to generate water, CO_2 and ATP.

In oxidative phosphorylation, a series of coupled reactions are involved in the transport of electrons through complexes 1-IV in the inner mitochondrial membrane. The entry point of electrons from the high energy molecules NADH and $FADH_2$ is at complex I and complex II respectively. With the help of a variety of enzymes, a series of coupled redox reactions drive the transport of electrons through these complexes. A proton gradient across the inner mitochondrial membrane is created during this process when protons are pumped out of the matrix at complexes I, III, and IV. The electrochemical gradient consisting of a pH gradient (ΔpH) and an electrical potential ($\Delta\psi$) drive the ATP synthesis from ADP as the protons re-enter the matrix through the ATP synthase (complex V).

Mitochondrial DNA (mtDNA) harboring their own genome with their own transcription, translation, and machinery for protein synthesis was discovered in the early 1960s. The codes for electron transport chain complexes, I, II, III, IV, and V for nuclear DNA (nDNA) are 36, 4, 10, 10, and 14 protein subunits and for mtDNA, 7, 0, 1, 3, and 2 subunits respectively [Carew & Huang, 2002]. Complex II is encoded by nDNA only. The mtDNA genome also encodes 22 mitochondrial tRNAs that are required for protein synthesis and 2 rRNAs that are essential for translation of mtDNA transcripts. The human mtDNA is a supercoiled, double-stranded molecule containing 16,569 base pairs [Chan, 2006; Carew & Huang, 2002].

A dynamic structural network consisting of about 70% stationary and 30% mobile mitochondria meets the energy demands of axons. The speed of the mobile mitochondria is ~ 1 μm/s [Kiryu-Seo et. al., 2010]. Mitochondrial fission and fusion, probable mitochondrial biogenesis within axons, and the transport of mitochondria to and from neuronal soma determine the content of mitochondria within axons.

Under highly reduced state of ETC, excess electrons at complex I produce $O_2^{\bullet -}$ in the mitochondrial matrix. This is reduced by the matrix MnSOD to H_2O_2. To a limited extent complex III also produces $O_2^{\bullet -}$ and is released into the mitochondrial intermembrane space where it is converted to H_2O_2 by Cu/ZnSOD. The presence of Fe^{2+} readily converts the H_2O_2 to the dangerous hydroxyl radical (reaction 7). The potential at the inner mitochondrial membrane, the pH in the matrix, local O_2 concentration and the nature of the

substrates dictate the amount of $O_2^{\bullet -}$ production. When the mitochondria is actively producing ATP, both ΔpH and Δψ as well as NADH/NAD+ ratio are low and ROS production is also low.

Superoxide production occurs on the outer mitochondrial membrane, in the matrix, and on both sides of the innermitochondrial membrane [Turrens, 2003]. The highly reducing intra-mitochondrial environment has reduced coenzymes and prosthetic groups of flavoproteins, iron-sulfur clusters (in Complex I) and ubisemiquinones (Complex III) that thermodynamically favor one electron reduction of molecular oxygen to produce $O_2^{\bullet -}$. While the major source of $O_2^{\bullet -}$ in the heart and lung is Complex III, it seems to be Complex I in the brain [Turrens, 2003]. Also the production of $O_2^{\bullet -}$ varies depending on the organ and whether the mitochondria is respiring or not. For example, in the absence of ADP, the proton movement through ATP synthase stops, protons build up and cause a slowdown of electron flow and thus creating a more reduced State IV respiration state. This reduced state and increasing concentration of O_2 will increase the one electron reduction process of oxygen and the rate of $O_2^{\bullet -}$ production will increase.

Uptake of high concentrations of Ca^{2+} into mitochondria, declined ATP, and increased ROS production endanger the health of the mitochondria and the mitochondria resort into a destructive mode by opening the mitochondrial permeability transition pore and consequently releasing cytochrome c and initiating programmed cell death.

6. Mitochondrial dysfunction in neurodegenerative diseases

Since different organs can rely on mitochondrial energy to different extents, mitochondrial defects can cause organ-specific phenotypes. The organ system most reliant on mitochondrial energy is the central nervous system. The consequences of mitochondrial dysfunction are numerous and include oxidative stress, loss of cellular Ca^{2+} homeostasis, promotion of apoptosis, and metabolic failure. Hence, evidence continues to accrue implicating mitochondrial dysfunction in the etiology of a number of neurodegenerative conditions such as Parkinson's, Alzheimer's, and transient ischemia.

In transient ischemia, a lack of oxygen and glucose delivery compromise the integrity of aerobic metabolism, while reperfusion potentiates injury via the generation of free radicals. Superoxide, nitric oxide and peroxynitrite production in the brain is increased during reperfusion following 30 minutes of global ischemia. In patients with Parkinson's disease, excess Fe^{2+} can reduce peroxide and produce $HO^{\bullet}$. These radicals and their reactions cause oxidative stress and consequent mitochondrial damage resulting in mutations. Evidence for mitochondrial dysfunction in Alzheimer's disease pathogenesis comes from impaired activities of three key Krebs Cycle enzyme complexes and reduced respiratory chain complex I, III, and IV activity observed in postmortem Alzheimer's disease brain, and oxidative damage to both mtDNA and nDNA. It may be possible for mtDNA mutations to disrupt the normal electron flow and seriously affect energy production. Oxidative damage and the resulting serious consequences have been extensively reviewed recently [Singh, 2006]. Compared to nDNA, mtDNA is far more susceptible to mutations due to their being present in a highly oxidative environment, a lack of protective histones and limited repair capacity [Carew & Huang, 2002; Singh, 2006].

During the production of ATP in the cell, about 85% of oxygen is consumed by the mitochondria. Superoxide radical, $O_2^{\bullet -}$, may be produced from about 4% of all oxygen

consumed [Singh, 2006]. Enzymes such as NADPH oxidases, xanthine oxidase, cyclooxygenases, and lipooxygenases also produce ROS. The iron-sulfur cluster in the aconitase enzyme, localized in the matrix space of mitochondria, is oxidized by superoxide and the exposed iron reacts with the peroxide to produce hydroxyl radicals [Singh, 2006]. Also the $NO^{\bullet}$ produced within mitochondria by mitochondrial NO synthase produces peroxynitrite [$ONOO^-$] by reaction with $O_2^{\bullet -}$. Superoxide radical and $ONOO^-$ contribute to substantial mitochondrial damage.

Enzymes such as SOD, GSHPx, catalase, peroxoredoxin, and thioredoxin can inactivate some of the ROS. MnSOD or Cu/ZnSOD converts the $O_2^{\bullet -}$ into H_2O_2. The active site of cytosolic and extracellular forms of SOD contains Cu/Zn and the mitochondrial form contains Mn [Beattie, 2002]. Oxidative damage is due to the inadequacy of these detoxifying processes.

In aging and neurodegenerative disorders, apart from inherited defects, mitochondrial DNA deletions and point mutations within neurons are well recognized.

Mitochondria is heavily involved in cell death. The various pathways involved in the cell death depend on the type of cellular injury or neurodegeneration. In the core region of stroke necrosis is observed. On the other hand in neurodegenerative diseases such as amyotropic lateral sclerosis, apoptosis markers are observed along with markers of endoplasmic reticulum(ER) stress and autophagy [Nagley et. al., 2010]. Mitochondria influences programmed cell death (type I-apoptotic as well as type III-necrotic). While autophagy routinely turns over various cellular constituents, it is involved in cell death (type II) in some stress conditions. Apart from changing the mitochondrial membrane potential and increasing the production of ROS, the elevation of intracellular and mitochondrial Ca^{2+} also modulates the process of programmed cell death. The involvement of mitochondria in the multifaceted neuronal death pathways is elegantly demonstrated in 4 steps in Fig. 1 [Nagley et. al., 2010]. Step A illustrates normal physiological conditions where equilibrium between homeostatic and deleterious factors is maintained. In this state of healthy functional neurons, cellular feedback mechanisms help maintain homeostasis. Step B is under conditions of minor stress. The deleterious factors such as reactive oxygen and nitrogen species (RONS) and misfolded proteins (MP) contribute to a decrease in energy production (ATP) and an increase in intracellular Ca^{2+}. Various channels and transporters elevate the Ca^{2+} in both cytoplasm and mitochondria. Neurons respond to this minor stress by activation of the unfolded protein response (UPR, in its initial, pro-survival phase), ubiquitin proteasome system (UPS), and chaperone-mediated autophagy (CMA). Step C indicates a much greater stress or imbalance due to substantial increases in RONS, MP, and Ca^{2+}. ATP production is substantially less. There is also a substantial decrease in mitochondrial membrane potential. The UPR switches to its destructive mode via induction of apoptotic effector proteins such as C/EBP homologous protein, caspase-12 and c-Jun N-terminal kinase. The UPS also becomes increasingly dysfunctional because of it s inability along with CMA to adequately handle the increased load of MP. This leads to the formation of intracellular aggregates of MP. Thus, deleterious factors overwhelm the cellular homeostatic processes. In spite of the chaperone mediated autophagic process switching to macroautophagy, it cannot adequately handle the load of MP leading to the final step D. There is a strong commitment to death at this level of stress and the cell advances to programmed cell death (type-I, type-II or type-III, or their combinations) [Nagley, 2010].

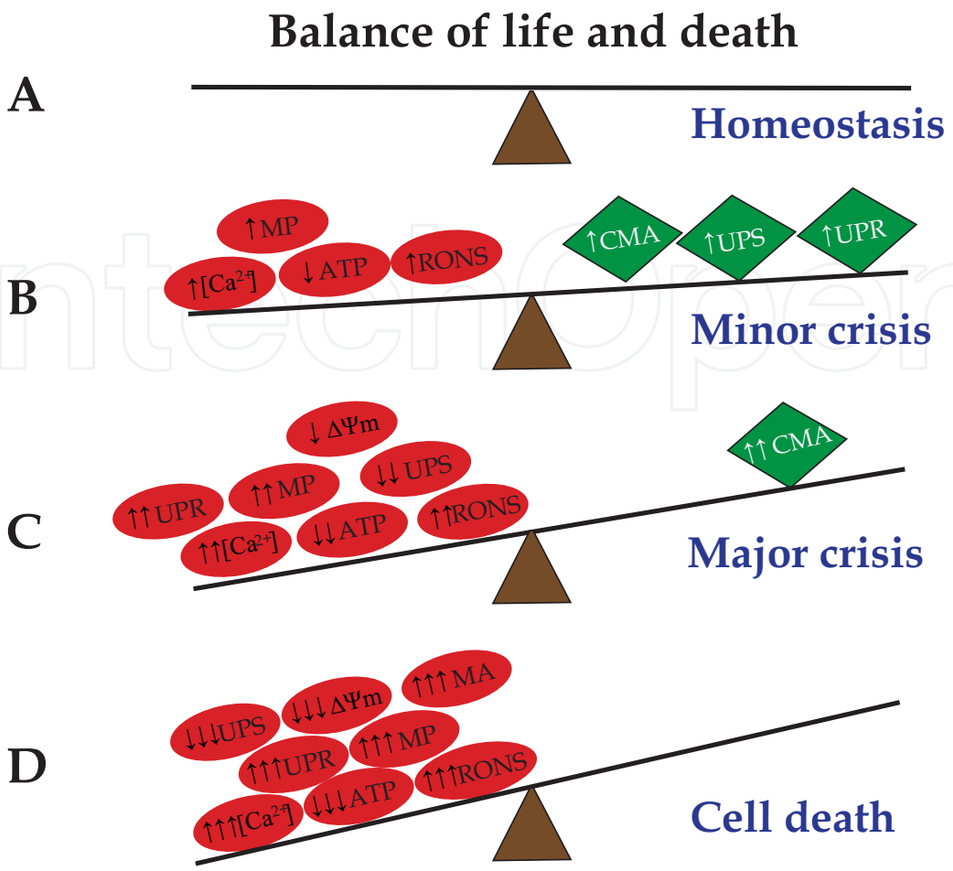

Fig. 1. Deleterious stress factors and restorative factors in neuronal homeostasis and neuronal death [Adpated from Nagley et. al., 2010]. $\Delta\Psi_m$, mitochondrial membrane potential; MP, misfolded proteins; RONS, reactive oxygen and nitrogen species; UPS, ubiquitin-proteasome system; UPR, unfolded protein response; CMA, chaperone mediated autophagy; MA, macroautophagy. Proteins failing to fold into native structure produces inactive and usually toxic proteins. Several neurodegenerative and other diseases are believed to result from misfolded proteins (MP). The unfolded protein response (UPR) is activated in response to a stress arising from an accumulation of unfolded or MP in the lumen of the endoplasmic reticulum. UPR attempts initially to restore the normal function of the cell by halting protein translation. They also activate the signaling pathways that lead to increasing the production of molecular chaperones involved in protein folding. If these efforts are not successful, then it induces apoptotic effector proteins. Proteasomes are very large protein complexes whose main function is to degrade unneeded or damaged proteins by proteolysis and thus helping cells to regulate proteins and MP. Ubiquitin, a small protein, is used to tag the proteins that need to be degraded. The overall system of ubiquitination and proteasomal degradation is known as the ubiquitin proteosome system (UPS). Autophagy, such as micro- and macro-autophagy, refers to the degradation of intracellular components via the lysosome. Chaperone mediated autophagy, CMA, can degrade only certain proteins and not organelles and thus very selective in what it degrades. Also the substrates are translocated across the membrane on a one on one basis instead of engulfing the substrate in bulk

7. Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system. It is associated with demyelination and a variable degree of axonal and neuronal degeneration. Demyelination decreases nerve impulse conduction velocity. Also the axons become vulnerable to inflammatory conditions. The mechanisms of tissue injury and neurodegeneration in MS are still under active investigation. Most MS patients initially experience relapsing-remitting episodes of neurologic deficits that last for six to eight weeks. The initial relapse rate is about 0.3/year. This rate declines progressively with time [Siffrin et. al., 2010]. This is followed by a gradual progression of irreversible neurological impairment or secondary progressive multiple sclerosis (SPMS) [Campbell et. al., 2011]. With advancing disease the observed increase in gray matter atrophy, which is indicative of the loss of neurons and axons, correlates well with the corresponding clinical disability.

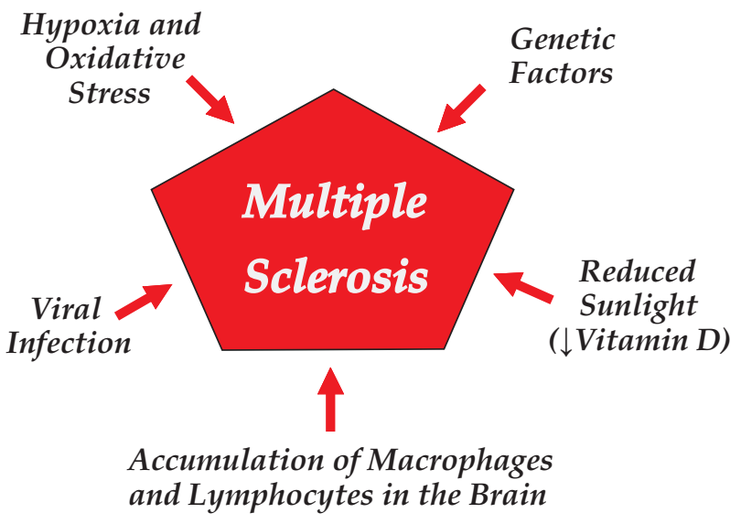

Fig. 2. Probable causal factors of multiple sclerosis [adapted from Mao & Reddy, 2010]

Apart from the consistent features, neuroaxonal injury and dysfunction in MS, the vascular aspects of MS, an increased risk for ischemic disease, global cerebral hypoperfusion, and a chronic state of impaired venous drainage are receiving a great deal of attention [D'haeseleer et. al., 2011; Filippi & Rocca, 2011].

The precise causal factors of multiple sclerosis are unknown. However, it is possible that multiple factors [Fig. 2] are involved in causing multiple sclerosis, including DNA defects in nuclear and mitochondrial genomes, viral infection, hypoxia and oxidative stress, lack of sunlight or sufficient levels of vitamin D, and increased macrophages and lymphocytes in the brain [Mao & Reddy, 2010].

Current research has shown that mitochondrial abnormalities are involved in the development and progression of multiple sclerosis [Fig. 3], including: mitochondrial DNA defects, abnormal mitochondrial gene expression, defective mitochondrial enzyme activities, abnormal or deficient mitochondrial DNA repair mechanisms, and mitochondrial dysfunction. Studies suggest that abnormal mitochondrial dynamics (imbalance in mitochondrial fission and fusion) plays a key role in tissues affected by multiple sclerosis. Furthermore, mitochondrial abnormalities and mitochondrial energy failure may impact

other cellular pathways including increased demyelination and inflammation in neurons and tissues that are affected by multiple sclerosis [Mao & Reddy, 2010].

While remyelination is extensive in some MS lesions, it is absent or incomplete in others [Zambonin et. al. 2011]. Remyelination helps to restore conduction and helps protect the axons from further inflammation. As an adaptive process or compensatory mechanism, demyelination in the central nervous system causes an increase in the mitochondrial content within axons [Mahad et. al., 2009; Kiryu-Seo et. al., 2010]. This has been attributed to the axons from further inflammation. As an adaptive process or compensatory mechanism, response to the changes in energy needs of axons caused by redistribution of sodium channels. Demyelination compromises the ionic balance and structural integrity of the axons. It results in diffusely expressed Na^+ channels with persistent Na^+ leakage and forces the need for additional energy to operate the Na^+/K^+ ATPase pumps. The mitochondria were found to increase in the order of increased energy demand, myelinated, remyelinated and demyelinated axons [Mahad et. al., 2009; Kiryu-Seo et. al., 2010; Zambonin et.al., 2011]. The increase in mitochondrial content (mostly stationary) within remyelinated compared to myelinated axons was attributed to the increase in density of the porin elements. (Porin is a voltage gated anion channel located in the outer membrane of all mitochondria). This increase in mitochondrial content resulted in a corresponding increase in mitochondrial respiratory chain complex IV activity. The change in demyelinated axons was attributed to the change in size. While the number of mobile mitochondria in both remyelinated and myelinated axons were nearly the same, they were much less in demyelinated neurons. An approximately 4 fold increase in mitochondrial content has been observed in chronically demyelinated and non-degenerative axons [Zambonin et. al., 2011]. However, increased mitochondrial content does not necessarily mean more activity. It has been demonstrated that while the mass number may increase in amyloid precursor protein positive segments of demyelinated axons, they appear to harbor mitochondria with complex IV defects [Mahad et. al., 2009]. Within injured axons (non-phosphorylated neurofilaments: SMI32) mitochondrial depletion and decreased complex IV activity was evident, in contrast to chronically demyelinated SMI31 positive axons located in the relatively inactive areas of chronic multiple sclerosis lesions exhibiting a significant increase in complex IV activity and mass [Table 1].

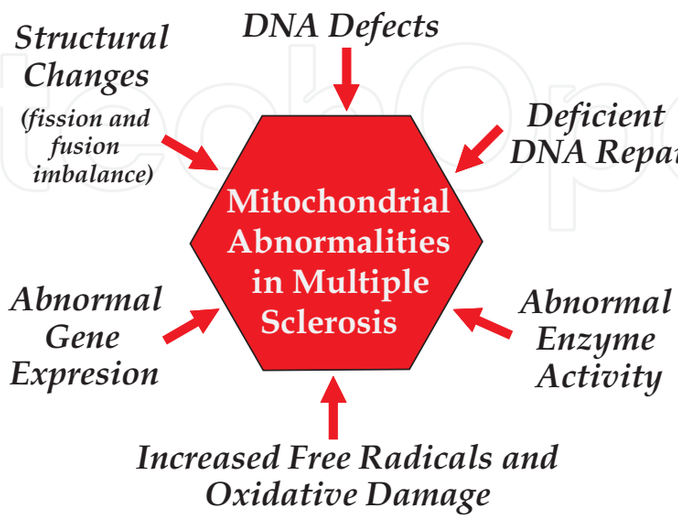

Fig. 3. Mitochondrial abnormalities in multiple sclerosis [adapted from Mao & Reddy, 2010]

	Control(SMI31)	NAWM(SMI31)	Lesion(APP)	Lesion(SMI32)	Lesion(SM131)
Complex IV					
Intensity					
Brian	17.93±5.89	16.14±6.54	9.85±6.94#	10.26±5.43#	20.10±7.38†, ‡
Spinal cord	16.99±15.49	16.87±12.44	9.01±11.16#	15.93±13.01	23.04±11.07*
Porin					
Percent Area					
Brian	6.74±.2.82	7.35±4.30	7.93±4.50	4.19±3.95†	11.15±5.23*
Spinal cord	5.93±4.38	3.46±3.92	8.26±7.40	1.64 ±1.39#	13.97±7.23*

Table 1. Quantification of the intensity of complex IV active elements and mitochondrial mass within axons [Mahad et. al., 2009]. The intensity of complex IV active elements represents the difference in densitometric value between background and complex IV active elements in inverted grey scale 100x brightfield images of cytochrome c oxidase or COX histochemistry. The percentage area of porin reactive elements within axons was calculated based on the total area of axonal porin reactive elements in triple labeled (porin, syntaphilin and axonal marker) and area of axons in confocal images. †P = 0.001 (versus NAWM) and ‡P = 0.002 (versus CON). #P<0.001 (versus SMI31 in CON, NAWM and lesion). *P<0.001 (versus SMI31 in NAWM and CON as well as SMI32 in lesion and APP in lesion). [For details, please see Mahad et. al., 2009]

Mitochondrial injury and subsequent energy failure have been implicated in the pathogenesis of MS [Lu et. al., 2000; Dutta et. al., 2006; Mahad et. al., 2008, 2009; Haider et. al., 2011; Campbell et. al., 2011]. Proteins and DNA in mitochondria are highly vulnerable to free radical damage and consequent mitochondrial injury in MS. The likely candidates involved in tissue injury in MS are the ROS and nitric oxide intermediates. These are produced by activated macrophages and microglia. In the brain tissue of patients with MS, oxidized DNA and oxidized lipids have been detected [Lu et. al., 2000]. Oxidized phospholipids and malondialdehyde (lipid peroxidation-derived structures) data from MS lesions of different activity of patients with acute, relapsing, remitting and progressive disease were found to be concentrated in active MS plaques, in areas known as initial demyelinating lesion or the "prephagocytic" stage of active MS lesions [Haider et. al., 2011]. There was good correlation between inflammation and the extent of DNA and lipid oxidation. Data in table 2 indicate up to a 5 fold increase in the extent of DNA damage (8-OHdG staining) and lipid oxidation (E06 and MDA-2 staining) in active lesions versus inactive lesions, normal white matter in MS patients, and white matter controls. The oxidation is predominantly seen in lesions with high T-cell and macrophage infiltrates and with profound microglial activation (HLA-D staining).

So far the efforts for complete restoration of axonal mitochondria following remyelination have not been successful. Thus the need for preservation of myelinated axons is exemplified by the fact that remyelinated axons have increased energy demand. This may also result in deficient neurons and reach detrimental levels in the long term. Mitochondrial DNA deletions have been found in the neurons in the progressive stage of MS [Campbell et. al., 2011]. The pathological features of MS lesions include demyelination and oligodendrocyte apoptosis, preferential destruction of small- caliber axons, differentiation arrest of oligodendrocyte progenitor cells and remyelination failure, and astrocyte dysfunction [Haider et. al., 2011].

	Active lesion	Slowly expanding Lesion	Inactive lesion	NAWM MS	White matter controls
	Median & (range)	Median & (range)	Median & (range)	Median & (range)	Median & (range)
Percentage of E06 positive area	3.1(26.5)	1.6 (16.7)	0.6 (9.2)	0.8 (17.1)	0.06 (2.9)
E06 axon spheroids	1.3 (18.2)	1.2 (121.2)	0.4 (2.3)	0.0 (1.2)	0.00 (0.0)
MDA-2 OG	1.6 (25.8)	0.9 (5.4)	0.9 (1.6)	0.4 (9.2)	0.00 (1.6)
8-OHdG nuclei	5.6 (78.1)	9.0 (19.0)	1.6 (6.8)	1.4 (12.0)	0.40 (10.4)
APP	44.3 (197.6)	16.1 (40.0)	0.3 (1.5)	0.0 (1.0)	0.00 (0.8)
CD3	73.6 (298.8)	51.2 (105.6)	10.0 (69.1)	4.8 (38.4)	9.60 (49.6)
HLA-D	324.8 (585.6)	148.3 (398.0)	29.0 (246.5)	99.3 (190.0)	43.10 (114.4)

Table 2. Quantification of oxidized lipids and oxidized DNA in different types of multiple sclerosis lesions in comparison with controls [Haider et. al., 2011]. Values represent medians and range (95th percentile range values); P-values are corrected by Bonferroni for multiple testing; all values represent counted cells/mm^2. Active lesions = classical actively demyelinating lesions; slowly expanding lesions = lesions with inactive lesion centre, surrounded by a small rim of activated microglia with recent myelin-degradation products; inactive lesions = lesions without any recent demyelinating activity; NAWM multiple sclerosis = normal-appearing white matter from patients with multiple sclerosis; white matter controls = normal white matter of all controls; APP = amyloid precursor protein reactive axonal spheroids or end bulbs; CD3 = T cells; percentage of E06 positive area = densitometric analysis of area covered by E06 immunoreactivity; E06 axon spheroids = axonal spheroids or end bulbs stained by E06 antibody; HLA-D = class II MHC-positive macrophages/microglia; MDA-2 OG = oligodendrocyte-like cells, immunoreactive for MDA-2; 8-OHdG nuclei = number of cell nuclei containing 8 hydroxy-D-guanosine immunoreactivity. Data show a highly significant accumulation of oxidized DNA and oxidized lipids in active multiple sclerosis lesions in comparison with controls. Oxidized DNA and lipids are predominantly seen in lesions with high T-cell and macrophage infiltrates and with profound microglia activation. (p values not included for brevity). [For details, please see Haider et. al., 2011]

A recent review details the current immunomodulatory treatments for MS. Other alternatives beyond immune-directed approaches are also speculated in this review [Aktas et. al., 2010]. Increased concentrations of reactive oxygen and nitrogen species found in MS, for example, lead to inhibition of ATP production within the axon. The ATP deficiency leads to loss of Na^+/K^+ ATPase and collapse of transmembrane ionic gradients. There is also an increase in intracellular Ca^{2+} levels and a decrease in mRNA levels of mitochondrial genes. At the same time immune –related demyelination also takes place. These results have prompted ion channel homeostasis as a potential therapeutic target to ameliorate the failed energy metabolism.

Peroxy nitrite formation at the site of inflammation has been measured using nitrotyrosine as a biochemical marker. Levels of nitrite and nitrate, the stable oxidation products of nitric oxide and peroxynitrite measured in cerebral fluid samples also revealed significantly higher levels of nitrate during clinical relapses of MS [Cross et. al., 1998].

In view of our data with palladium α-lipoic acid formulation (section 10) on the enhanced enzymatic activities of Krebs cycle and electron transport chain enzymes in animals, anti-oxidant activity and the ability of this formulation to repair DNA, we had decided, as a preliminary step, to investigate its usefulness in ameliorating the fatigue conditions in 15 MS patients. The study is expected to be completed soon.

8. Cerebral ischemia

An insufficient or reduced blood flow to the brain to meet the metabolic demand will result in cerebral or brain ischemia. The normal cerebral blood flow is ~ 50 to 60 mL/100g/min. Death of brain tissue is a consequence of poor oxygen supply or cerebral hypoxia resulting from the insufficient blood flow. A prototype of brain damage during cerebral ischemia is shown in Fig. 4. [adapted from Mehta et. al., 2007]. Maximum damage occurs as a result of ischemic necrosis (infarction) at the "core" or "focal" tissue region, where the blood flow is < 7 mL/100g/min. Since the cellular integrity is compromised during necrosis, cellular damage repair at the core is extremely hard. The blood flow in the surrounding less-severely ischemic boundary ("penumbral" or "perifocal" tissue) is ~ 7 to 17 mL/100g/min. The penumbra is metabolically active but electrically silent [Mehta et. al., 2007]. More moderate alterations develop in this region because of the near normal glucose use, but the oxidative metabolism is still impaired. Different mechanisms contribute to cell death in the core and penumbra due to differences in the severity of ischemia.

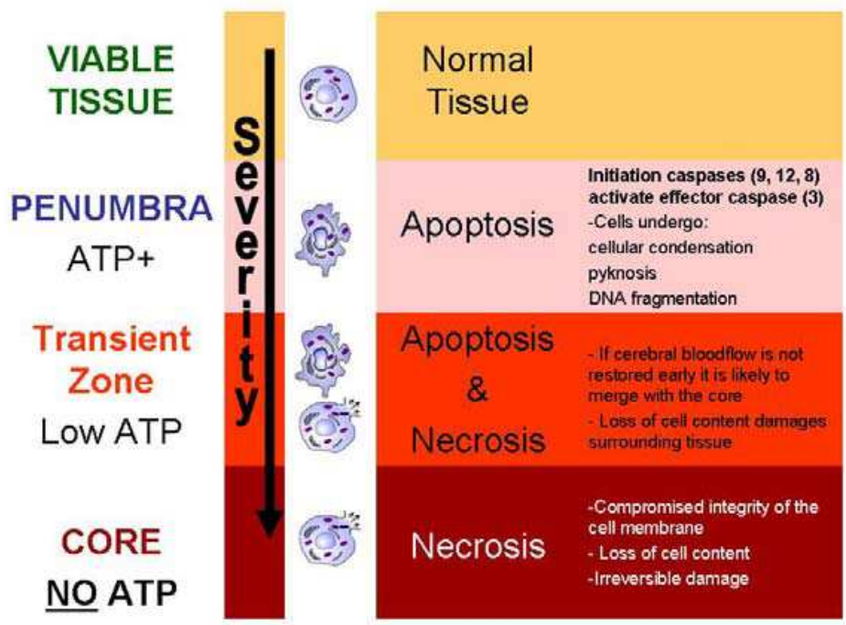

Fig. 4. A prototype brain damage during cerebral ischemia: core, a region where cells undergo necrosis. The region surrounding the core is called ischemic penumbra, a site of delayed mode of cell death (apoptosis) due to availability of ATP. Further, a transient zone in-between the core and penumbra is likely to merge to the core if the cerebral blood flow is not restored early. The penumbral region is surrounded by a region of viable tissue [adapted from Mehta et. al., 2007]

Therapeutic quick intervention may reduce the infarct volume in the penumbra because the irreversible damage occurs relatively slowly [Sims & Muyderman, 2010]. The only embolic or thrombolytic agent used to reverse arterial occlusion within the first 3 h is tissue plasminogen activator (tPA). As an enzyme, it catalyzes the conversion of plasminogen to the enzyme responsible for clot breakdown, plasmin. In ~17 % of ischemic stroke patients spontaneous reversal of occlusion takes place in 6 h and in 40-50% of patients in 4 days. Quick restoration of normal blood flow to this region can result in substantial recovery of energy- related metabolites. However, in the post ischemic tissue, energy requirements are low and the glucose oxidation is due to limitations on the mitochondrial oxidation of pyruvate. This results in a secondary impairment of mitochondrial function and consequent cell death. Normalcy of function can be maintained in the outermost viable tissue.

While focal ischemia is confined to a specific region of the brain, global ischemia encompasses wide areas of the brain tissue. Focal ischemia occurs in a region when a blood clot has occluded in a downstream region of artery in the brain (ischemic stroke). This occlusion or blockage may be caused by thrombosis (a blood clot formed locally obstructing the blood flow) or arterial embolism (obstruction of blood flow due to an embolus from elsewhere in the body). While ischemic strokes are caused by interruption of the blood supply, hemorrhagic strokes are the result of rupture of a blood vessel. Hemorrhagic strokes result in areas of friable tissue, containing areas of both viable and dead tissue. Transient global ischemia involves a brief interruption in blood flow usually in a larger cerebral vessel, e.g. middle cerebral artery, resulting in primarily apoptotic cell death.

More than 80% of all strokes are due to focal ischemia. Unless treated, the occlusion of an artery produces tissue infarction resulting in a loss of all cells including neurons, astrocytes, oligodendrocytes, microglia and endothelial cell [Sims & Muyderman, 2010]. The stroke results in mitochondrial impairment because the blood flow becomes < 20% of the normal and a consequent reduction in glucose and oxygen supplies ensue. This attenuates ATP and reactive oxygen species production as well as apoptosis. Lack of ATP production disrupts the ionic gradients across the plasma membrane. The net result is marked losses of intracellular K^+ and a large influx of Ca^{2+} into cells [Doyle et. al., 2008]. Thus there is heavy involvement of the impaired mitochondria in the development of the tissue injury after ischemic attack, due to modifications in ATP production and other mitochondrial changes leading to apoptosis and necrosis.

Further, a transient zone in-between the core and penumbra is likely to merge to the core if the cerebral blood flow is not restored early. The damage in this transient zone is a result of the release of cellular contents from those necrotic "core" cells, such as the oxidative enzymes of various organelles i.e. lysozomes and peroxisomes. Eventually this transient zone would contribute to the total infarct volume. The penumbral region is surrounded by a region of viable tissue [Mehta et. al., 2007]. Penumbra varies in size and can be rescued

Ischemic cell death is also attributed to abnormal activation of enzymes such as poly-ADP ribose polymerase (PARP) and the caspases. Oxidative stress, which produces free radical nitric oxide ($NO\cdot$) and reactive peroxynitrite ($ONOO^-$), is implicated in both necrosis and apoptosis in focal ischemia. Peroxynitrite is formed by the reaction of $NO\cdot$ with superoxide. Mitochondria are targeted by peroxynitrite and the resulting mitochondrial dysfunction during severe hypoxia-ischemia increases generation of oxygen free radicals. This leads to dysfunction of cellular membrane causing necrosis [Mehta et. al. 2007]. An additional consequence of ischemia involves the dissociation of the electron transport chain within minutes of the insult. Ubiquinone and cytochrome C, which serve as electron shuttles,

translocate from the inner mitochondrial membrane. This is of particular consequence upon restoration of blood flow. While reperfusion limits some damage, oxidative stress is increased under these conditions. It has been found that over expression of Mn^{2+}-superoxide dismutase, which converts superoxide to hydrogen peroxide results in moderate reductions in the size of infarction in temporary ischemia [Sims & Muyderman, 2010]. Addition of the mitochondrial uncoupling agent, dinitrophenol, was found to modulate the Ca^{2+} content and production of free radicals in the mitochondria of penumbra [Korde et. al., 2005].

The reduced delivery of oxygen and glucose to the tissue in focal ischemia affects the function of the mitochondria. Mitochondrial properties undergo further changes depending on the severity and duration of ischemia and also following reperfusion. Development of cell death pathways depends on the impaired mitochondria's ability to generate ATP.

	Focal ischemia		**Reperfusion**	
	Core	Penumbra	Core	Penumbra
Metabolites				
ATP	↓↓↓	↓↓	↓↓	↓
Adenylate energy charge	↓↓	↓	↓/N.C.	N.C.
Total adenine nucleotides	↓↓	↓↓	↓↓	↓
Phosphocreatine	↓↓↓	↓	↓	N.C.
Lactate	↑↑	↑↑	↑↑	↑/N.C.
Glucose	↓↓↓	N.C.	N.C.	N.C.
Metabolic activity				
Glucose use	↓↓↓*	N.C.	↓↓	↓↓
Oxidative metabolism	↓↓↓*	↓↓↓	↓↓	↓↓

Table 3. The effects of focal ischemia for up to 2 h and of subsequent reperfusion for 1 h on the content of energy-related metabolites and pathways of energy metabolism [Sims & Muyderman, 2010]. Differences are shown compared to non-ischemic tissue. ↓: decreased to >65%; ↓↓: decreased to between 35% and 65% ; ↓↓↓: decreased to less than 35%; ↑: increased less than four-fold; ↑↑: increased greater than four-fold; N.C.: no significant change. Two symbols indicate findings that differ between published reports. *, direct evaluation of these properties in severely ischemic tissue may not give reliable information. The magnitude of these reductions is assumed from the large decrease in substrate delivery and large changes in ATP and phosphocreatine content [Sims & Muyderman, 2010]

The changes in energy-related metabolites and in the contributing metabolic pathways in brain tissue in the first 2 h of ischemia and reperfusion for 1 h are summarized in Table 3 [Sims & Muyderman, 2010]. In the core, the ATP and glucose content falls significantly in the first 5 min of occlusion and then ATP stabilizes to ~ 15-30% of normal for at least the first 2 h and then reaches about 50%. The initial rapid decrease is attributed to the major redistribution of ions across the plasma membrane of cells. In view of our admittance data on the H_2O_2-Ca^{2+}, H_2O_2-Na^{+} interactions, we strongly believe that ion-peroxide interactions must also be playing a major part in this process. The adenylate energy charge, a measure of intracellular balance between ATP, ADP, and AMP, is given by:

$$\text{Adenylate energy charge} = \{[ATP] + 0.5\,[ADP]/\,[ATP] + [ADP] + [AMP]\} \quad (31)$$

The adenylate energy charge decreases rapidly to ~ 0.4-0.5 during the initial hours and remains above 0.8 after 2 h compared to the normal value in the brain of ~ 0.93. While adenylate kinase catalyses the conversion of some ADP to AMP to meet short term energy needs of the brain, in ischemic tissue, the adenine nucleotide pool is depleted by the conversion of AMP to inosine and hypoxanthine. Phosphocreatine, the short term energy reserve of the brain falls quickly to <30% of normal during ischemia. Phosphocreatine stabilizes to about 70% of normal after ~ 2 h of ischemia. ATP regeneration from ADP is catalyzed by the enzyme creatine kinase. Lack of oxygen forces glucose to go the glycolytic pathway creating a 10-fold increase in lactate and consequent lowering of pH. Of course lack of removal of lactate due to limited blood flow may also be contributing to this accumulation. In addition, restricted blood flow appears to have a greater effect on the delivery of oxygen to the tissues versus glucose, since penumbral glucose levels are either the same or slightly higher, while lactate levels are much higher (but less than in the core).

During the first 2 h following reperfusion, phosphocreatine and adenylate energy charge are recovered to >90% of normal compared to ATP values of 50-70%. This resistance of ATP for restoration is attributed to the depletion of the adenine nucleotide pool. In the penumbral tissue, phosphocreatine and adenine nucleotide balance, but not ATP are recovered almost completely within 1 h of reperfusion for ischemic periods of 3 h or longer [Sims & Muyderman, 2010]. The glucose utilization is less in the penumbral region during the first hour of reperfusion. The lactate, on the other hand, is decreased during this period.

The reduction in ATP production in the ischemic brain may be associated with decreased neuronal activity of the post-ischemic brain as a result of the enzyme AMP-activated protein kinase enzyme [Sims & Muyderman, 2010].

It must be mentioned that there is a complete or near complete recovery of mitochondrial respiratory function in core and penumbral tissues within the first hour following reperfusion. This is followed by a secondary deterioration, indicative of the development of irreversible cell dysfunction.

Under normal physiological conditions, the channel within the N-methyl-D-aspartic acid (NMDA) glutamate receptor is blocked in a voltage dependent manner by Mg^{2+}. The triggering of energy deficits and neuronal depolarization are the results of decreased cerebral blood flow. The mild depolarization results in the dislodging of Mg^{2+} and glutamate, which are consequently released in large amounts to the extracellular space. This leads to an over-activation of the NMDA and AMPA glutamate receptors. Since these receptors regulate Ca^{2+} ion channels, a calcium ionic imbalance occurs in neurons. This influx of Ca^{2+}, is due to this increase in glutamate release from neurons and astrocytes induced by the ischemia. Apart from the traditional ionotropic glutamate receptors, the influx of Ca^{2+} is also attributed to some emerging metabotropic and channel mechanisms that include: sodium-calcium exchangers, hemi channels (unopposed half-gap junctions), acid sensing channels, volume-regulated anion channels, nonselective cation channels and signaling cascades that mediate crosstalk between redundant pathways of cell death [Besancon et al., 2008]. This abnormal intracellular accumulation of Ca^{2+} is involved in the triggering of cell death by up regulation of a wide range of cell death executioners that include ATPases that serve to further deplete energy stores, lipases that damage lipid membranes of organelles and the cell surface itself, proteases that dismantle the cytoarchitecture of the neuron, and DNAses that damage the nucleus [Besancon et. al. 2008].

Bioenergetics of cerebral ischemia (both focal and global) as well as gray and white matter ischemia were recently reviewed from a cellular perspective. A brief summary is given in

Table 4 [Hertz, 2008]. During the early stages of ischemia, fatal injury is observed for neurons and oligodendrocytes. They are very sensitive to the excitotoxicity of glutamate due to their cell process expression of NMDA receptors and cell body expression of AMPA/kinase receptors. Astrocytes and endothelial cells seem to survive longer. Neurons are damaged from lack of astrocyte support. Axonal injury is due to channel mediated Na^+ uptake followed by Na^+/Ca^{2+} exchange.

	Neurons	Axons	Oligodendrocytes	Astrocytes	Endothelial cells
Intracellular Na^+ increase	X	X	X	X	X
Increased metabolism	X	?	?	X	?
Intracellular Ca^{2+} increase	X	X	X	X	?
Mitochondrial damage	X		X	X	?
Formation of ROS	X		X	(X)	X

Table 4. Bioenergetic mechanisms involved in ischemic death of different cell types and constituents [Hertz, 2008]

Studies have demonstrated that ischemic damage may be reduced by blockade of ionotropic glutamate receptors using glutamate receptor antagonists [Mehta et. al., 2007; Besancon et. al., 2008; Doyle et. al., 2008; Sims & Muyderman, 2010]. The most extensively evaluated neuroprotectors that include calcium channel blockers, glutamate antagonists, GABA agonists, antioxidants and radical scavengers, and $NO\cdot$ signal down regulator, have been critically reviewed recently [Ginsberg, 2008].

Other neuroprotective approaches involve the use of anti-oxidants. As an example, α-lipoic acid reduced the mortality rate of male Sprague-Dawley rats from 78% to 26% during 24 hours of reperfusion. It was found effective in improving survival and protecting the rat brain against reperfusion injury following cerebral ischemia [Panigrahi et. al., 1996]. In another study rats that received subcutaneous treatment of R-or S-lipoic acid for 2 hours before ischemia significantly reduced the infarct volume [Wolz & Krieglstein, 1996]. Similar results with mice were obtained with 100 mg/kg of lipoic acid given subcutaneously 1.5 hours before ischemia [Clark et. al., 2001]. Transient global ischemia also benefits from pretreatment with α-lipoic acid. Administration of 40 mg/kg for 7 days protected from ischemic damage when gerbils were tested for locomotor behavior and morphological damage to the CA1 region of the hippocampus [Cao & Phillis, 1995].

Animal studies, using adult male Mongolian gerbils, used as controls or treatment group with palladium α-lipoic acid complex formulation (PdLA), demonstrated that acute, post ischemic and prophylactic administration of PdLA limits ischemic damage [Antonawich et. al., 2004]. Following bilateral carotid artery occlusion in the gerbil, the PdLA was administered intraperitoneally (IP) immediately after surgery, then once daily for 3 days. The control group received saline. PdLA treatment significantly protected hippocampal pyramidal cells (CA1) from transient global ischemia at 30, 50, and 70 mg/kg per 24 h.

While a delayed application of the palladium α-lipoic acid complex formulation after 48 hours of ischemic attack had no significant effect in protecting CA 1 cells, a delayed administration after 6 hours of ischemic attack was as good as giving it immediately after ischemic attack in minimizing cell death.

Five minutes of carotid artery occlusion was sufficient to hinder the characteristic nesting behavior of gerbils for ~ 3 days. Their nesting behavior was observed to improve significantly after treatment with palladium lipoic acid complex formulation (50 mg/kg every 24h and 30 mg/kg /24h at 24 and 72 hours after ischemia. The lack of nesting behavior at 70 mg/kg-treated animals was attributed to their excessive energy and consequent ignoring of the nesting material.

It was observed that preventive or prophylactic treatment with 10 mg/kg gerbil (or allometric scaling equivalent of 10 mL-human dosage) offered significant behavioral and morphological improvement from transient global ischemia.

Our studies demonstrate a greater protective effect of palladium α-lipoic acid complex versus α-lipoic acid alone (Cao & Phillis, 1995). Four times more α-lipoic acid and for a longer period of pre-treatment were necessary to obtain morphologiocal protection. Further more immediate administration of palladium α-lipoic acid complex formulation protected over 70%of the CA1 neurons, and administration delayed up to 24 hours after the TIA still offered significant protection (30% of the CA1 pyramidal cells) [Antonawich et. al., 2004].

9. The powerful super-antioxidant, α-lipoic acid

Alpha- lipoic acid is a very unique and simple biological molecule. It has a carboxylic acid group with a pK_a of 4.7. It is ionized at biological pH, and it has a cyclic disulfide or dithiolane ring [Baumgartner et. al., 1996; Patel & Packer, 2008]. It exists intracellularly as the reduced form, (±)-dihydrolipoic acid. Lipoic acid occurs naturally as a coenzyme in both prokaryotic and eukaryotic cells, as well as in plants, and animals including humans. It is enzymatically synthesized from octanoic acid in the mitochondrion.

α-Lipoic acid, absorbed intact from the diet, is readily converted into dihydrolipoic acid in many tissues. In the intracellular environment, two or more enzymes reduce the exogenous lipoic acid. The reversible reduction to dihydrolipoic acid is favored by the presence of the ring strain in the 1, 2-dithiolane ring of about 15-25 $kJmol^{-1}$ [Baumgartner et. al., 1996]. α-Lipoic acid exits as R(+)-and S(-)- enantiomers due to the presence of an asymmetric carbon. The biologically active enantiomer is mostly the former one. Since its first isolation in 1951, numerous investigations have been carried out to decipher the uniqueness of this simple but elegant molecule [Reed 2001; Patel & Packer, 2008].

Molecular mechanisms and therapeutic potential of α-lipoic acid, a dietary supplement, have been reviewed recently [Shay et. al., 2009]. Lipoic acid can cross the blood-brain barrier. The biological effects of lipoic acid are attributed to its redox property, the antioxidant capacity and the fatty acid properties. There is ample evidence indicating the usefulness of the lipoic acid/dihydrolipoic acid redox couple as a therapeutic agent for diabetes, ischemia-reperfusion injury, heavy metal poisoning, modulator of various inflammatory signaling pathways, age associated cardiovascular, cognitive, and neuromuscular deficits, protection from radiation damage, neurodegeneration, and HIV infection [Packer et. al., 1995; Patel & Packer, 2008; Shay et.al., 2009]. Dihydrolipoic acid can regenerate or recycle the antioxidants CoQ (ubiquinol), vitamins C and E (via glutathione), and glutathione without itself becoming one in the process. Dihydrolipoic acid also prevents lipid peroxidation by regenerating glutathione. [Packer et. al., 1995; Patel & Packer, 2008].

Lipoic acid and dihydrolipoic acid are efficiently transported in and out of both mitochondria and cells. Compared to this, the transport of disulfides such as cystine that is needed in modulating glutathione (GSH) levels in cells is very inefficient. The mitochondrial

β-oxidation of lipoic acid has been attributed to its fatty acid properties, similar to that of octanoic acid [Patel & Packer, 2008]. Redox as well as biological antioxidant effects have been attributed to the β-oxidation products of lipoic acid, the oxidized and reduced forms of bisnorlipoic acid and tetranorlipoic acid [Patel & Packer, 2008].

Oxidant	Scavenged or not by LA and rate constant	Scavenged or not by DHLA and rate constant
Peroxynitrite	Yes, 1.4×10^3 M^{-1} S^{-1}	Yes, 2.5×10^2 M^{-1} S^{-1}
Nitric oxide	No	Yes, 3.19 M^{-1} S^{-1}
Hydroxyl radical	Yes, 4.7×10^{10} M^{-1} S^{-1}	No
	Yes	Yes
Superoxide	No	No
	Yes, 3.3×10^5 M^{-1} S^{-1}	
		Yes
Singlet oxygen	Yes, 1.3×10^8 M^{-1} S^{-1}	No
	Yes	
Peroxyl radical	Yes, 1.8×10^8 M^{-1} S^{-1}	Yes, 2.3×10^7 M^{-1} S^{-1}
	No	Yes
Hypochlorous acid	Yes	Yes
Hydrogen peroxide	No	No

Table 5. Antioxidant activities of lipoic acid (LA) and dihydrolipoic acid (DHLA) [detailed references in Shay et. al., 2009]

The α-lipoic acid/dihydrolipoic acid couple is called a "universal antioxidant" because it fulfills several criteria used to evaluate the antioxidant potential as well as preventive or therapeutic applications of a compound such as specificity of free radical quenching, metal chelating ability, interaction with other antioxidants, effects on gene expression, absorption and bioavailability, concentration in tissues, cells, and extracellular fluid, and location (in aqueous or membrane domains or in both) [Packer et.al., 1995].

The free radical scavenging activities of lipoic acid and dihydrolipoic acid are given in Table 5 [Shay et.al., 2009]. Both are capable of scavenging peroxynitrite, peroxyl radical and hypochlorous acid, but not hydrogen peroxide. There are conflicting data for singlet oxygen and radicals such as hydroxyl, superoxide, and peroxyl.

Questions have been raised regarding the direct-acting antioxidant status of LA/DHLA in vivo based on the invitro data [Shay et.al., 2009]. This is due to the large dosage, 200-600 mg, used for invivo studies and the amounts found in the plasma (both area under the curve and C_{max} are in the range of microgram to nanogram levels per mL). The administration of lipoic acid in acid form or salt form, oral or intravenous, with a meal or without meal also contributed to the fluctuations in the data. Also the invitro data cannot imitate the invivo clearance of 98% of LA excretion in the urine within 24 hours.

α-Lipoic acid was found to protect hematopoietic tissues in mice from radiation damage [Ramakrishnan et. al., 1992]. It was also found that α-lipoic acid offered protection from radiation for children affected by the Chernobyl nuclear accident [Korkina et al., 1993]. α-Lipoic acid scavenges hydroxyl radicals but is not effective against hydrogen peroxide

and superoxide radical. The reduction potential for the α-lipoic acid/dihydrolipoic acid couple of -320 mV or -290 mV [Krishnan et. al., 2011] and the GSSG/GSH) couple of -240 mV indicate that dihydrolipoic acid can react with GSSG and regenerate GSH [Packer et. al., 1995]. Thus lipoic acid helps to maintain GSH/GSSG ratio (about 100 to 10,000 times greater than other redox couples such as NAD^+/NADH, and $NADP^+$/NADPH), an estimate of redox state, in cells [Patel & Packer, 2008].

Treatment with lipoic acid increases the GSH levels in human cell lines and primary cells including T cells, erythrocytes, lymphocytes, and glial and neuroblastoma cells.. This is explained by 1) facile transport of lipoic acid into cells, where it is reduced by NADH or NADPH dependent pathways to dihydrolipoic acid. 2) Dihydrolipoic acid is transported back into the extracellular media where it is oxidized by cysteine regenerating lipoic acid and producing cysteine, the limiting substrate on GSH synthesis. 3) Compared to cystine, cysteine is more easily transported into the cell and aids the synthesis of GSH [Patel & Packer, 2008].

Thus elevated levels of GSH and ascorbic acid, which in turn regenerates vitamin E, are all indicative of lipoic acid acting as an inducer of endogenous antioxidants. It has been reported that lipoic acid is also an effective regulator of signaling pathways and induces synthesis of GSH transcriptionally. Lipoic acid reverses the decline in transcriptional activity of Nrf2 caused by age-related loss of GSH [Suh et. al, 2004].

The pharmacokinetics of R-lipoic acid, reviewed recently [Patel & Packer, 2008; Shay et.al., 2009], revealed a plasma level concentration, C_{max}, of 1.154 μg/mL from 1 g R-lipoic acid compared to the proposed therapeutic range of 10-20 μg/mL or 50-100 μM (Carlson et al., 2008). A dose of 600-800 mg sodium R-lipoate gave plasma levels of 8-18 μg/mL, which is within the therapeutic range. The upper limit suggested for therapeutic action of 45 μg/mL or 225 μM is reached by a dose of about 1.2 g of racemic-α-lipoic acid. The no adverse observed effect level (NOAEL) of racemic lipoic acid is considered to be 60 mg/kg body mass/day. Therapeutic and energy production applications of this powerful antioxidant have been explored extensively [Patel & Packer, 2008].

Located within the mitochondrial matrix are lipoic acid requiring enzymes: three α-keto acid dehydrogenase complexes that catalyze the oxidative decarboxylation of α-keto acids such as pyruvate, α-ketoglutarate, and branched chain α-ketoacids [Voet D. and Voet J. G., 1995]. In organisms, hydrogen atom transfer and acyl group transfer take place in the oxidative decarboxylation of α-ketoacids with the aid of α-lipoic acid. The reversible redox reaction between α-lipoic acid and dihydrolipoic acid is thus a very important biochemical reaction. The reversible reduction to dihydrolipoic acid is favored by the presence of the ring strain in the 1,2-dithiolane ring of about 15-25 $kJmol^{-1}$ [Patel & Packer, 2008].

The multienzyme complex, pyruvate dehydrogenase, consists of three enzymes, pyruvate dehydrogenase (E1), dihydrolipoyl transacetylase (E2), and dihydrolipoyl dehydrogenase (E3) [Voet D. and Voet J. G., 1995]. This enzyme complex participates in five sequential reactions during the conversion of pyruvate to acetyl-CoA. The R- lipoic acid is covalently linked to a ε-amino group of lysine residue via an amide linkage. These lipoic acid containing enzymes participate in four out of the five reactions.

The multienzyme complex, α-ketoglutarate dehydrogenase, also consists of three enzymes, α-ketoglutarate dehydrogenase (E1), dihydrolipoyl transsuccinylase (E2), and dihydrolipoyl dehydrogenase (E3) [Voet D. and Voet J. G., 1995]. The branched chain α-ketoacid dehydrogenase is also a multienzyme complex resembling the other two enzymes

mentioned above. These three enzymes have the same dihydrolipoyl dehydrogenase and employ the coenzymes thiamine pyrophosphate, lipoamide, FAD and the terminal oxidizing agent NAD^+ [Voet D. and Voet J. G., 1995]. The importance of lipoic acid in the energy metabolism is illustrated by these three enzymes.

Disulfide bonds in proteins are formed between the thiol groups of cysteine residues. The other sulfur-containing amino acid, methionine, cannot form disulfide bonds. More aggressive oxidants convert cysteine to the corresponding sulfinic acid and sulfonic acid. A variety of oxidants promote this reaction including air and hydrogen peroxide. Such reactions are thought to proceed via sulfenic acid intermediates.

The oxidized form α-lipoic acid can undergo further oxidation at sulfur or get reduced. This property, which is somewhat similar to an intermediate oxidation state in a transition metal or a nonmetal, makes this molecule very unique compared to other biological molecules. A biological oxidation product of α-lipoic acid (lipoic acid S-oxide or a thiolsulfinate) is known as β-lipoic acid or Protogen-B [Baumgartner et. al., 1996]. It has not been possible to conclusively prove which sulfur is oxidized [Stary et. al. 1975].

The oxidation of α-lipoic acid was found to be a one-electron charge transfer process, pH independent, and an irreversible process [Corduneanu et. al., 2009]. Most voltammetric studies were centered on the oxidation of α-lipoic acid and studies related to its reduction to dihydrolipoic acid were limited [Rogers & Mallett, 1983]. To understand the electrochemical behavior of α-lipoic acid, we had explored potential regions beyond the normal range of the mercury electrode, both on the cathodic side as well as on the anodic side. The advantage in using the mercury electrode is the ease with which we can obtain a fresh consistent surface and drop. We have not observed (visually) any passivation of mercury on the anodic side when lipoic acid was present without any background electrolyte. We had investigated the cathodic side, the normal potential region for mercury working electrode, in much greater detail.

The complexity of the redox process of α-lipoic acid is shown in the cyclic voltammograms in Fig. 5 [Krishnan & Garnett, 2011]. We had assigned the three cathodic peaks to 1) reduction of lipoic acid S-oxide, 2) reduction of lipoic acid to most probably dihydrolipoic acid, and 3) probable reduction of lipoic acid dimers or higher polymers. We have assigned the five anodic peaks to 4) the oxidation product of lipoic acid dimers or higher polymers, 5) oxidation product of dihydrolipoic acid, 6) formation of S-oxide of lipoic acid dimers or higher polymers, 7) formation of lipoic acid S-oxide, and 8) further oxidation of lipoic acid S-oxide/the formation of thiolsulfonates. The peak due to the formation of lipoic acid S-oxide (peak 7) is missing in Figure 3a because of the formation of S-oxide of lipoic acid dimers or higher polymers (peak 6). However, when the formation of lipoic acid polymers is minimized by restricting the scan to less cathodic potentials (to -0.5 V instead of -2.0 V) the formation of S-oxide of lipoic acid dimers or higher polymers is minimized and thus allowing the formation of lipoic acid S-oxide (peak 7) [Krishnan & Garnett, 2011]. Other complexities were expressed at differing scan rates, at higher concentrations and by the presence of electrolytes. The data shown in Fig. 5 are for 1mM α-lipoic acid where solute-solute interactions are minimized and in the absence of electrolytes so that the influence of ion-dipole interactions can be more readily investigated at the double layer.

The molecule, α-lipoic acid, is very unique because of its ability to form a variety of radicals, dimers and higher polymers as well as a variety of lipoic acid S-oxides. This complexity is reflected in its electrochemical redox behavior. Our data demonstrated that depending on

the concentration of lipoic acid and the scanning potential, radicals and polymers are formed [Krishnan & Garnett 2011]. Also our data suggest the need for a revalidation of the reported redox potential of the lipoic acid/dihydrolipoic acid couple.

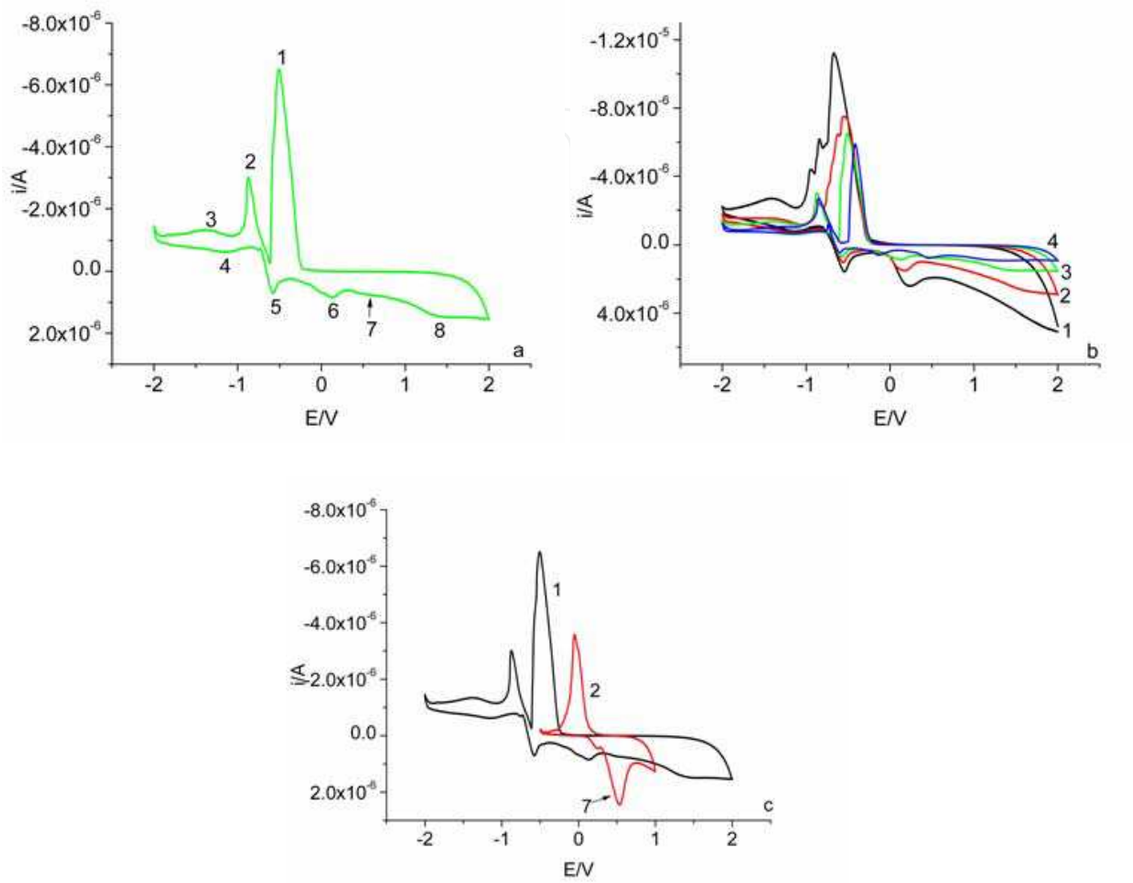

Fig. 5. Cyclic voltammogram of 1 mM α-lipoic acid (sodium salt), pH 7.2, scan 2.0 to -2.0 V and back, scan 3, scan rate a and c, 100mV/sec; b. scan rates 1) 400 2) 200 3) 100 4) 50 mV/sec; **c.** 1) scan 2.0 to -2.0 V and back, 2) scan 1.0 to -0.5V and back; peak 7 same as peak 7 in a. [Krishnan & Garnett 2011]

Our electrochemical data also suggested a caution in deciding on the dosage of oral supplements of lipoic acid because of its tendency to form dimers and higher polymers under reducing biological conditions. The pharmacokinetic data mentioned earlier is also subject to these complexities depending on the dosage chosen for the studies.

10. Catalytic, electronic, and therapeutic properties of palladium α-lipoic acid complex

The coordination chemistry of palladium complexes have recently been reviewed with an emphasis on cancer therapy[Gao et. al., 2009]. Even though there are many structural and thermodynamic similarities between the complexes of palladium and platinum, the palladium(II) complexes seem to exhibit biological action very different from those of the

toxic platinum complexes. Copper, zinc, and arsenic complexes of α-lipoic acid and palladium α-lipoic acid complexes with 1:1 and 1:2 stoichiometry have been reported [Garnett, 1995a, 1995b; Strasdeit et. al., 1995; Baumgartner et. al., 1996].

Palladium α-lipoic acid complex has demonstrated numerous antitumor activities against various cell lines. Also it was found to halt the growth of glioblastoma in nude mice. Clinical veterinary studies indicated its effectiveness as a complimentary support to chemotherapy. A recent Phase I, dose escalation study, has revealed safety in humans up to 40 mL 0.037 M of this complex with no severe adverse events, and minor adverse events i.e. mild gastrointestinal irritation, and aversion to taste. Washout periods for palladium, monitored in blood serum and urine, ranged from three to seventeen weeks after cessation of the formulation [Krishnan et. al., 2011].

The electrochemical characteristics of α-lipoic acid and palladium α-lipoic acid complex have been explored using glassy carbon and highly oriented pyrolytic graphite electrodes [Corduneanu et al., 2007, 2009]. Palladium complex was found to dissociate at negative potentials with deposition of Pd(0) nanoparticles. The application of a positive potential induced the oxidation of the palladium complex and the formation of a mixed layer of lipoic acid and palladium oxides.

Superior free radical scavenging capacity, as measured by oxygen radical absorbance capacity or ORAC analysis, was observed for palladium α-lipoic acid complex formulation (5.65) compared to vitamin E (1.0, normalized value), vitamin A (1.6), vitamin C (1.12), and α-lipoic acid (1.4) [Krishnan et.al., 2011]. This may be compared to the superior antitumor activities observed for metal complexes compared to that of their ligands [Matesanz et. al., 1999; Maloň et. al., 2001].

The enhanced energy effects observed in gerbils during transient ischemia studies [Antonawich et. al., 2004] prompted us to investigate the influence of palladium α-lipoic acid complex on the activities of enzymes involved in mitochondrial energy production. The activities of four Krebs cycle enzymes, isocitrate dehydrogenase (ICDH), α-ketoglutarate dehydrogenase (α-KGDH), succinate dehydrogenase (SDH), and malate dehydrogenase (MDH) and mitochondrial complexes I, II, III, and IV in aged male albino rats were investigated [Sudheesh et. al., 2009]. The enhanced activity of the metal complex was superior to the activity of lipoic acid, the ligand.

The antioxidant status in the heart of aged male Albino Wistar rats, measured by MnSOD, CAT, and GSHPx, were higher with the palladium lipoic acid treated group than with the α-lipoic acid group [Sudheesh et al., 2010]. This was also true in alloxan induced diabetic rats [Sudheesh et. al. 2011]. Similarly the lipid peroxidation levels were lowered and the GSH levels were increased in the palladium α-lipoic acid treated group. It is not clear at this time whether scavenging some free radicals by either α-lipoic acid or palladium α-lipoic acid complex formulation is connected in any way to the enhanced activities of Krebs cycle and mitochondrial enzymes.

A specific example is illustrated here. The α-ketoglutarate dehydrogenase complex (KGDH) is a critical component of Krebs cycle and of glutamate metabolism. Glutamate is an excitotoxic neurotransmitter. Reactive oxygen species modify the activity of KGDH. It is also known that the activity of KGDH is lower than that of any other enzyme in the brain. Deficiencies in KGDH lead to brain neurological syndromes. Palladium α-lipoic acid complex increases the activity of KGDH and thus helps in the removal of the glutamate.

The antioxidant status in the liver, kidney and brain of mice after exposure to 2-6 Gy radiation were also measured recently [Menon et. al., 2009]. The results were similar to the ones observed in the heart of rats without radiation. Also an analysis of their blood leukocytes and bone marrow for DNA damage using alkaline single cell gel electrophoresis (alkaline comet assay) revealed DNA repair from the lowering of comet assay parameters, DNA in tail, tail length, tail moment, and olive tail moment. Administration of the complex also reduced the mortality of the mice and also aided recovery from the radiation induced weight loss [Ramachandran et. al., 2010]. DNA repair was also observed in human blood leukocytes with palladium α-lipoic acid complex treatment immediately after exposure to radiation [Menon & Nair, 2011].

The mechanism of the superiority of the metal complex compared to that of the ligand is still an unsolved puzzle. Compared to the high oral dose, the available plasma concentration of α-lipoic acid is very small. One possibility for the higher activity of the metal complex is probably due to its increased concentration in the plasma. This needs experimental verification. It is also known that 98% of α-lipoic acid is excreted in urine within 24 hours and since it takes 4-6 weeks for serum and urine clearance of palladium, the previous suggestion seems justified. Another possibility is the chemistry of the transition metal playing a dominant role in the enzymatic activity. The starting material in the synthesis of the palladium α-lipoic acid complex is palladium(II). The final complex is also palladium(II), based on preliminary ESR data. Palladium(II) complexes are diamagnetic. The complex concentration dependent electrochemical characteristics of α-lipoic acid suggest the possibility of free radical formation by one electron reduction under physiological conditions. In such a case the electron spin may be involved in the enzymatic process. The impedance characteristics of the palladium α-lipoic acid as well as that of the α-lipoic acid, described in this section, strongly suggest this possibility.

Another possibility for the superiority of the palladium α-lipoic acid complex compared to α-lipoic acid is its ability to form self-assembled structures, such as the one shown in Fig. 6. No self-assembly was observed for sodium lipoate. We want to point out that binding of a lysine residue in the protein to the lipoyl group of E2 in 2-oxoacid dehydrogenases results in a long flexible arm that can oscillate a distance of ~200 Å. This arm is utilized during the catalytic cycle [Patel & Packer, 2008]. It is obvious that the self assembled palladium α-lipoic acid complex can make this process more facile.

The physics and chemistry of non-equilibrium systems have been utilized to understand some of the spatial patterns and temporal patterning observed in biological processes such as bacterial colonies shaped by diffusive instabilities and calcium waves governed by nonlinear amplification during intracellular signaling [Levine & Jacob, 2004].

In homogeneous systems, spiral waves and spatiotemporal phenomena are formed from autocatalytic reactions and diffusion resulting from chemical instabilities. Our data suggest that the propagation of electrical signaling among the packing units and extending to long distances by global coupling is viable by such self-assembled systems.

We have utilized the technique of electrochemical impedance spectroscopy extensively to probe spatiotemporal phenomena in biological systems. This technique is routinely used to study corrosion and fuel cells. We have used admittance measurements for understanding solute-solvent interactions, "π –way" conduction, ion pair formation, water-structure enforced ion pair formation, potential induced and solvent mediated ion pair formation at the double layer, and semi conduction characteristics of simple biological molecules

[Krishnan & Garnett, 2006; 2011; Krishnan et al., 2007a,b; 2008a,b,c,d; 2009a,b; 2011]. Simple molecules such as arginine, histidine, lysine, flavin adenine dinucleotide, riboflavin, cysteine, lidocaine hydrochloride, α-lipoic acid, and hydrogen peroxide exhibit negative differential resistance, a characteristic of a tunnel diode.

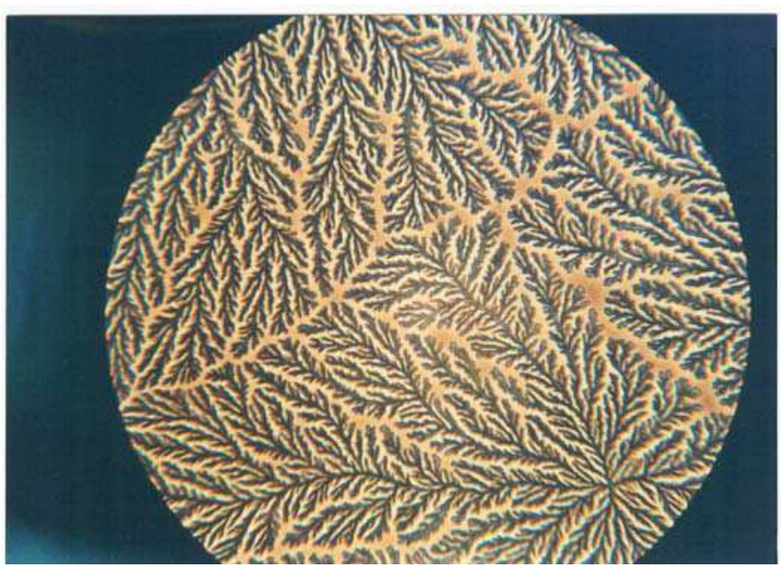

Fig. 6. Phase microscopy of palladium α-lipoic acid complex, 300X [Krishnan & Garnett, 2006]

In this technique a perturbing sinusoidal voltage $E = E_o\sin(\omega t)$ is applied at angular frequency ω ($2\pi f$, where f is the conventional frequency in Hz) to the electrode system consisting of a working electrode, counter electrode and reference electrode. The measurements reported in this chapter were made using and EG & G PARC Model 303A SMDE trielectrode system (mercury working electrode, platinum counter electrode and Ag/AgCl saturated KCl reference electrode) along with Autolab ecochemie. The measurements were carried out in the range 1000Hz to 30 mHz. The amplitude of the sinusoidal perturbation was 10 mV. The response of the applied sinusoidal voltage is analyzed in terms of the resultant current $I = I_o\sin(\omega t + \Phi)$, where Φ represents a characteristic phase angle shift. In the plane of Cartesian coordinates, an impedance is expressed by its real (Z′) and imaginary (Z″) parts. The modulus $|Z|$ and phase angle Φ of $\mathbf{Z}(\omega)$ can be obtained from $|Z| = [Z'^2 + Z''^2]^{1/2}$ and $\Phi = \tan^{-1}[Z''/Z']$, respectively [Macdonald & Johnson, 2005]. Admittance and impedance are interrelated:

$$Z'/Y' = Z''/Y'' = (Z')^2 + (Z'')^2 = 1/[(Y')^2 + (Y'')^2 \quad (32)$$

Over a frequency bandwidth of interest, there are various ways of representing the impedance spectrum. Most often, the well known Nyquist or Cole-Cole plot (Z″ as the Y-axis and Z′ as the X-axis for the range of frequencies explored at a fixed potential) and Bode plot ($|Z|$ and Φ vs. $\log\omega$) are employed to represent the data. In simple terms, impedance is like a frequency dependent generalized resistance and admittance is like a frequency dependent conductance. In electrochemistry, the imaginary impedance is almost always capacitive and therefore negative. Majority of impedance data require only the first

quadrant in the plot. However, if there is inductance, the data will require the first and fourth quadrants. In corrosion studies, the oxides formed at passivation potentials exhibit, quite often, semiconduction characteristics and their impedance data will be in both first and second quadrants. The impedance in the second quadrant may be compared to the negative differential resistance (NDR) observed in the I-V curves of tunnel diodes and some enzymes. Impedance spectra spanning more than two quadrants and especially four quadrants are unusual and are often explained by nonequilibrium phenomena and compared to spatiotemporal oscillations in biological systems.

The impedance spectra for α-lipoic acid and its modulation by complexing with palladium are shown in Fig. 7. While α-lipoic acid exhibits NDR and shows impedance in only 3 quadrants (chaotic in quadrant 3), the spectra of the metal complex is extended to 4 quadrants and much more smoothly by complexation with palladium. Of course the NDR behavior can be optimized by slightly tweaking the applied potential. This enhancement in NDR behavior may be compared to the enhanced Krebs cycle and mitochondrial enzymatic activities of the palladium α-lipoic acid compared to that of the ligand.

We have reason to believe that the self-assembled structure of the complex, by providing a spatial extension of the membrane with much more surface area, may be catalyzing the electron transfer process by enhancing spin coupling. This may account, for example, the enhanced complex I and complex II activities activities of PdLA by 151% and 212% more than that of α-lipoic acid.

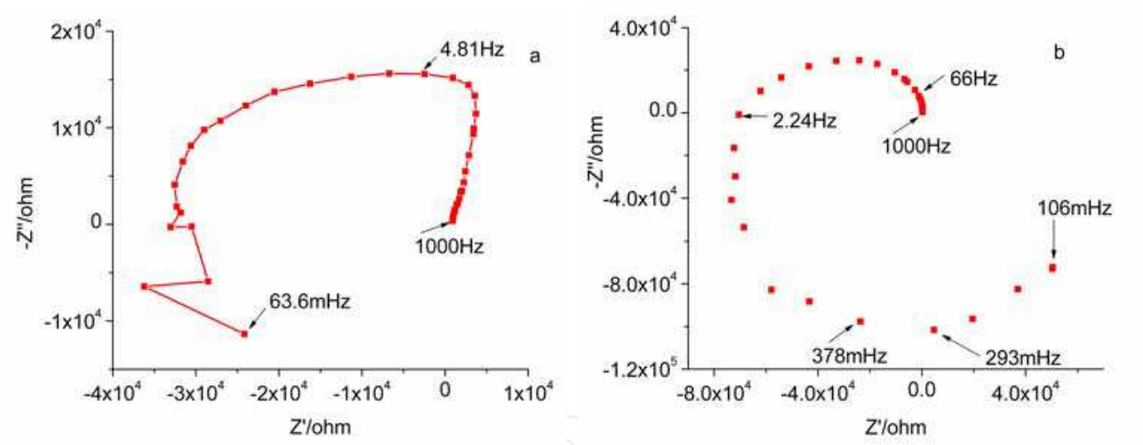

Fig. 7. a) Nyquist plot for 0.0373 M sodium lipoate, -1.15V, pH 7.79, NDR at 4.81Hz.
b) Modulation of lipoate impedance by palladium in 0.0373M palladium α-lipoic acid (1:1 complex) in 0.1792 M NaCl, -1.18V, pH 7.78, NDR at 66Hz [Krishnan et. al., 2011]

Another important aspect of this system is the fact α-lipoic acid is linked to lysine by an amide bond in the multienzyme complexes of pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and branched chain α-ketoglutarate dehydrogenase. Thus both α-lipoic acid and lysine have heavy involvement in the electronic aspects of the enzymatic process.

11. Conclusions

Oxidative stress is caused by the chemical imbalance between ROS production and their breakdown by antioxidants. Over-abundance of ROS has been found during neuronal

development, as well as in numerous neuropathological conditions. Oxidative stress and mitochondrial dysfunction have been closely associated in brain injury such as ischemia and stroke and neurodegenerative processes such as Multiple Sclerosis, Parkinson's, Alzheimer's, and Huntington's.

Lipoic acid was found to be effective in modulating many neurodegenerative disorders. The development of palladium α-lipoic acid complex was intended to augment the properties of this ligand by the catalytic properties of the transition metal. It is formulated to combat mitochondrial dysfunction. The unique electronic properties of palladium modulating the properties of α-lipoic acid appear to be a key to this physiological effectiveness. This is exemplified in our electrochemical impedance spectroscopic studies of α-lipoic acid and palladium α-lipoic acid complex.

Palladium α-lipoic acid complex facilitates aerobic metabolism much more than that of α-lipoic acid, by significantly enhancing the enzymatic activity of isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase at the Krebs cycle and mitochondrial complexes I, II, III, and IV of the electron transport chain. The electronic properties of palladium also appear to modulate the antioxidant properties of α-lipoic acid in that PdLA enhances the activities of catalase and glutathione peroxidase more than that of α-lipoic acid. The level of GSH also was significantly improved and the level of lipid peroxidation was decreased in the heart mitochondria of aged rats.

PdLA is similar to a multi-spectrum drug. Since it targets the mitochondria it is able to carry out several functions such as combating age-related as well as disease-associated fatigue, and minimizes the effects of ischemic injury. Being a powerful free radical scavenger, it may also be effective in combating death of neurons and other progressive loss of structure or function of neurons caused by free radicals.

PdLA is able to protect from radiation exposure and repair DNA. It also seems to ward off radiation exposure-associated weight loss in mice, possibly protecting susceptible gastrointestinal tract.

12. Acknowledgements

The area covered in this chapter is vast and warrants citation of numerous publications. The authors regret being unable to cite many publications due to space limitations. The authors wish to express their sincere thanks and gratitude for many fruitful discussions and collaborations in various aspects of the work presented in this chapter: B. Chu, K. K. Janardhanan, T.A. Ajith, C.K.K. Nair, N.P. Sudheesh, A. Menon, and L. Ramachandran.

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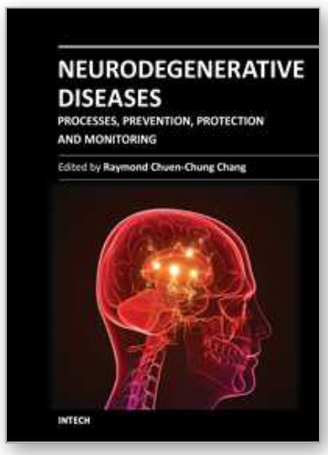

Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring

Edited by Dr Raymond Chuen-Chung Chang

ISBN 978-953-307-485-6
Hard cover, 558 pages
Publisher InTech
Published online 09, December, 2011
Published in print edition December, 2011

Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring focuses on biological mechanisms, prevention, neuroprotection and even monitoring of disease progression. This book emphasizes the general biological processes of neurodegeneration in different neurodegenerative diseases. Although the primary etiology for different neurodegenerative diseases is different, there is a high level of similarity in the disease processes. The first three sections introduce how toxic proteins, intracellular calcium and oxidative stress affect different biological signaling pathways or molecular machineries to inform neurons to undergo degeneration. A section discusses how neighboring glial cells modulate or promote neurodegeneration. In the next section an evaluation is given of how hormonal and metabolic control modulate disease progression, which is followed by a section exploring some preventive methods using natural products and new pharmacological targets. We also explore how medical devices facilitate patient monitoring. This book is suitable for different readers: college students can use it as a textbook; researchers in academic institutions and pharmaceutical companies can take it as updated research information; health care professionals can take it as a reference book, even patients' families, relatives and friends can take it as a good basis to understand neurodegenerative diseases.

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