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UNDERSTANDING THE MECHANISM OF OXIDATIVE STRESS GENERATION BY OXIDIZED DOPAMINE METABOLITES: IMPLICATIONS IN PARKINSON'S DISEASE

by

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DISSERTATION

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DEDICATION

To my Parents and my Grandparents

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For the past two years I have been battling with the question, "do you see light at the end of the tunnel?" Every time, I answered "may be," but eventually the light turned out to be another set of hurdles. Finally, I can say confidently that 'yes,' I can see happiness at the end of the tunnel as my PhD comes to a satisfying end. Certainly this wasn't a 'one man show' and I would like to take this opportunity to thank every single person who has positively contributed towards this rollercoaster journey of mine.

Firstly, of course, my advisor Dr. David Njus. He has been a pillar of support and a stellar guiding force all through these six and a half years. He has not only encouraged my new ideas but has also given me enough space to execute them. I have seen my friends go through pressure and anguish in dealing with their PIs. Thankfully the situation for me was exactly the opposite. His cheerful attitude towards life and kind nature made him even more friendly and helpful. I wonder if he even knows how to get angry. Also unlike other PIs, he actually encouraged me to go to India and visit my family, which meant a lot to me. He is the best mentor I could have asked for. His penchant for science and zealous approach towards it has inspired me to work towards achieving my goals and fulfilling my dreams in the scientific world. Thank you Dr. Njus for everything. And thank you for making my life so very comfortable. You are a good soul. I will miss working with you.

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Special thanks to my dearest friend Ashlesha. She has been a great support to me and has really helped me in more than many ways. Her commitment for science is infectious and her honesty for work, incredible. She has inspired me a lot and pushed me to do my best work. Thank you for always motivating me. One day I hope to be as good a scientist as you.

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LIST OF ABBREVIATIONS

PD: Parkinson's Disease

DA: Dopamine

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MPP+: 1-methyl-4-phenyl-2,3-dihydroxypyridinium ion

DAT: Dopamine transporter

ROS: Reactive oxygen species

RNS: Reactive nitrogen species

GSH: Reduced glutathione

GSSG: Oxidized glutathione (glutathione disulfide)

TH: Tyrosine hydroxylase

L-DOPA: L-3,4-dihydroxyphenylalanine

AADC: Aromatic amino acid decarboxylase

VMAT2: Vesicular monoamine transporter 2

HOCD: Hypochlorite oxidized cysteinyl-dopamine

cysDA: Cysteinyl-dopamine

PARP: Poly-ADP-ribose polymerase

MPO: Myeloperoxidase

NADPH: Nicotinamide adenine dinucleotide phosphate (reduced)

NADH: Nicotinamide adenine dinucleotide (reduced)

3-MAQ: 3-methyl-5-anilino-1,2-benzoquinone

DTT: Dithiothreitol

6-OHDA: 6-Hydroxydopamine

DOPAL: 3,4-dihydroxyphenylacetaldehyde

ALDH: Aldehyde dehydrogenase

H_2O_2: Hydrogen peroxide

CHAPTER 1 – INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative movement disorder characterized by specific loss of dopaminergic neurons in the substantia nigra pars compacta region of the midbrain. In the year 1817, James Parkinson originally described this disorder in a systematic manner as 'shaking palsy' (1). He described the symptoms of the disease in his early paper titled, "An Essay on the Shaking Palsy," as "involuntary tremulous motion with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured" (1). Until 1877, the condition continued to be referred to as 'paralysis agitans,' when French neurologist Jean Charcot coined the term "Parkinson's Disease" (2). However, it was not until 80 years later that the characteristics that eventually defined the disease came to the forefront during anatomical investigations that showed brain lesions in the post-mortem mid-brains of PD patients (2). Over the years, unraveling the underlying cellular mechanisms that particularly render the dopaminergic neurons vulnerable has been a subject of intense research.

Following Alzheimer's disease, PD is the second most common neurological disease in both the USA and other industrialized nations, affecting over 1 million people in the USA itself (3). Apart from the specific degeneration of dopamine neurons, formation of protein aggregates called Lewy bodies is another major pathohistological hallmark commonly associated with the disorder (4). Lewy bodies are cytoplasmic inclusion bodies primarily containing intraneuronal protein deposits such as α-synuclein, ubiquitin,

neurofilaments and tau protein (5). Recent articles have suggested that such protein aggregates are not restricted to the substantia nigra or midbrain (6).

Loss of dopamine (DA) neurons primarily affects the motor functions leading to symptoms such as bradykinesia, resting tremor, rigidity and postural instability among others (3). Accompanying motor deficits are also non-motor issues such as sleep disturbances, depression, anxiety and cognitive impairments (7,8).

Epidemiological studies have revealed that most PD cases (90%) are sporadic and have a late onset with the median age of onset being around 65 years (9). Such cases that do not have a known genetic basis are also termed as idiopathic forms of PD. The remaining ~10% of cases are characterized by early onset and are typically associated with familial forms of PD (10). Development of Parkinsonian syndrome in such individuals has been attributed to mutations in several identified genes. Some of the genes known to be involved in such familial forms are α-synuclein (SNCA), ubiquitin C-terminal hydrolase (UCH-L1), parkin (PRKN), leucine rich repeat kinase-2 (LRRK-2), phosphatase and tensin homologue induced putative kinase 1 (PINK1), C57 peptidase (PARK7 or DJ-1), and probable cation-transporting ATPase 13A2 (ATP13A2) (11,12). Recent evidence however has suggested genetic predisposition as a risk factor for non-familial forms of PD (13).

Parkinson's disease represents a final outcome that involves complex interactions between multiple factors which surround the innate vulnerability of dopaminergic neurons in the nigro-striatal pathway. There are a number of factors known to contribute to neurodegeneration, such as dopamine oxidation (14,15), oxidative stress (16-18), mitochondrial dysfunction (19-21), genetic defects (11,12) and microglial inflammation

(22,23). Although multiple players have been implicated in the progression of the disease, the specific mechanisms connecting these disparate aspects have been elusive.

Environmental factors such as pesticides and toxins directly induce both oxidative damage and mitochondrial dysfunction (24,25). Occupational uses of herbicides, exposure to organic solvents, carbon monoxide, and carbon disulfide, and more generally, industrialization, agricultural environment, well water, plant-derived toxins and bacterial and viral infection are all suggested to play roles (26-30). A number of cellular toxins are known to induce high-affinity specific inhibition of mitochondrial complex I or proteasomal inhibition, viz., rotenone, paraquat, epoxomicin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). These toxicants have been popular study models for Parkinson's disease due to their ability to mimic the specific loss of dopamine neurons (31-33). MPTP is known to cross the blood brain barrier easily after its systemic administration and gets converted to an actively toxic metabolite 1-methyl-4-phenyl-2,3-dihydroxypyridinium ion (MPP+) by the enzyme monoamine oxidase-B (MAO-B). The metabolite MPP+ is selectively taken up into dopaminergic neurons by the dopamine transporter (DAT), where it is thought to irreversibly inhibit complex I of the mitochondrial respiratory chain, thereby producing selective degeneration of nigral dopamine cells (34-36). Rotenone, on the other hand, is a well-known universal inhibitor of the mitochondrial respiratory chain. It inhibits the transfer of electrons from complex I to ubiquinone (37). What makes the rotenone model particularly interesting is, although it also leads to the formation of Lewy bodies, it is still not known how rotenone specifically targets dopaminergic neurons.

Mitochondrial dysfunction has long been implicated in the etiology and pathogenesis of Parkinson's disease. Apart from defective respiration, perturbations in mitochondrial dynamics such as alterations in mitochondrial morphology and intracellular trafficking, are also involved. Chronic depletion of antioxidant defenses such as glutathione due to mitochondrial complex I impairment is suggestive of the interplay between oxidative stress and mitochondrial dysfunction. Mitochondrial damage may result from oxidative stress and, vice versa, its impairment may also enhance ROS/RNS release into the cytosol (38,39).

Oxidative stress is also one of the major pathological factors involved in Parkinson's disease. It is defined as a condition where the formation and accumulation of reactive oxygen and/or nitrogen species are favored over their removal, due to the defense mechanisms being overwhelmed. Factors such as proteasome pathology, microglial activation, neuro-inflammation and mitochondrial dysfunction, also identified as potential candidates in PD, have been closely associated to oxidative stress. Analysis of postmortem brains from PD patients revealed enhanced oxidative stress in the nigrostriatal dopaminergic neurons with a decreased ratio of GSH/GSSG (40), augmented levels of iron (41), increased lipid peroxidation (42), elevated keto-protein formation (43) and DNA oxidation (44). A relation has been shown in various animal models and in brains taken from deceased PD patients between neuronal destruction in the brain and the generation of massive oxidative stress. Betarbet et al. developed an *in vivo* rotenone-treated rat model for Parkinson's disease (45). The rotenone-treated rats exhibited chronic inhibition of complex I of the mitochondrial electron transport chain in the brain. Moreover, the model exhibited highly specific neurodegeneration of nigrostriatal

dopaminergic neurons along with the symptoms characteristic of PD – Lewy body formation, motor deficits, hypokinesia and rigidity.

Of late, the role of oxidative stress in Parkinson's disease has been highly controversial. A major contention has been that the appearance of the oxidative biochemical markers may be due to some other causative factor involved in neuro-degeneration, rather than the reverse (46,47). Thus the question whether oxidative stress is involved as the cause or consequence of the neurodegeneration of PD is still wide open. Also, the fact that many non-dopaminergic pathways are affected in the process has brought into question the significance of dopamine oxidation as the causative agent of oxidative stress. Therefore, it is imperative to differentiate between the cause and the consequence, to elucidate the mechanism underlying dopaminergic cell death in order to understand the etiology of Parkinson's Disease.

Dopaminergic neurons comprise 1% of the total brain neurons, but they control numerous functions in the brain. Some of the critical functions where dopaminergic neurons play a role are movement, motivation and reward, learning, emotion, cognition and pain pathways (48). They originate in the ventral tegmental area, in the substantia nigra and in the hypothalamus. These dopamine neurons project their axons to other areas of the brain building a complex neuronal network encompassing four major pathways. First, the mesolimbic pathway that connects the ventral tegmental area to the limbic system, controls memory and motivating behaviors. Second, the mesocortical pathway projects to the frontal cortex and surrounding structures. Third, the tuberoinfundibular pathway secretes hormones such as prolactin by connecting the

hypothalamus to the pituitary gland. And fourth, the nigrostriatal pathway, in which axons project from the substantia nigra to the striatum, controls the motor functions.

The synthesis of dopamine takes place in the cytoplasmic compartment by the action of tyrosine hydroxylase (TH) on the amino acid tyrosine. In this hydroxylation step, tyrosine hydroxylase is a monoxygenase enzyme that uses tyrosine and molecular oxygen as substrates and tetrahydrobiopterin and iron as co-factors. This is the rate limiting step in the biosynthetic pathway of dopamine. The resulting product, L-3,4-dihydroxyphenylalanine (L-DOPA), is then decarboxylated by aromatic amino acid decarboxylase (AADC) to dopamine. Once dopamine is formed, it is rapidly sequestered into synaptic vesicles by the action of vesicular monoamine transporter 2 (VMAT2), where low pH prevents its auto-oxidation. When dopamine is released in to the synaptic cleft, the dopamine transporter (DAT) ensures rapid DA reuptake (49). If free dopamine levels in the cytosol or synaptic cleft exceed transport capacity, it can be metabolized via enzymatic or non-enzymatic oxidative pathways.

Studies with animal models, neuronal cell lines and also various post mortem brain studies have hinted at the potential role of dopamine and/or its metabolites as endogenous neurotoxic agents, influencing redox balance, ROS production and oxidative stress (14,15,50,51). This may be due to redox cycling of catechols, leading to increased generation of detrimental ROS. The catechol ring (ortho-dihydroxybenzene) of dopamine contributes significant toxicity to its structure. Hasegawa et al. showed that oxidized catechol metabolites cause apoptotic death of dopaminergic neurons and this process was exacerbated by alpha-synuclein, a major constituent of Lewy bodies (15). Moreover,

these metabolites could form adducts with alpha-synuclein leading to α-synuclein oligomerization, disruption of mitochondrial membrane potential and cell death (52).

The oxidation of dopamine gives rise to quinone formation, both semi-quinone as well as ortho-quinone. DA oxidation can result in the formation of radicals (semi-quinones) and quinones via one- and two-electron oxidations, respectively (53-57). This process is enhanced in the presence of metal ions such as iron, copper, or manganese by Fenton chemistry leading to increased production of reactive oxygen/nitrogen species. Research has shown that dopamine cytotoxicity is primarily due to the formation of dopamine quinone (54,58), which is more powerful and toxic than quinones produced from other catecholamines. Also, intrastriatal injection of dopamine to normal rats in a dose-dependent manner results in formation of lesions in the corpus striatum that was found to be mediated by quinone formation (58,59). The dopamine quinone also reacts with the sulfhydryl group of cysteine possibly leading to protein modification (55,60,61). Thus, these oxidized dopamine metabolites create a potentially damaging environment for dopaminergic neurons.

The cells affected in PD are centers of dopamine (DA) synthesis, storage, and metabolism. The concentration of dopamine and its turnover within the neurons of the nigro-striatal pathway is very high (48). Consequently, these neurons are under an immense oxidative stress threat even in their resting state. Thus the intracellular metabolism of dopamine is a potential source of oxidative stress, capable of exerting toxicity by directly damaging cellular macromolecules such as proteins and DNA, and depleting oxidative defenses through redox cycling (57,62,63).

Therefore, the intent of our research is to study the redox cycling mechanism of dopamine oxidized metabolites and investigate the mechanism by which they cause oxidative stress. Our hypothesis is that the death of dopaminergic neurons in Parkinson's disease is caused by oxidative stress created by redox cycling of dopamine oxidation products. To test this hypothesis, I pursued the following specific aims:

I. How do redox cyclers compare in different modes of redox cycling?

II. Does dopamine oxidize to form cytotoxic compounds capable of redox cycling?

III. What is the underlying mechanism of cell death?

CHAPTER 2 – MATERIALS AND METHODS

Oxygen consumption assay for redox cycling

Redox cycling was measured at 37ºC in 4 ml of 50 mM potassium phosphate, 1 µM EDTA, pH 7.4 using a YSI Model 5300A Clark-type oxygen electrode. O_2 concentration was calibrated using glucose and glucose oxidase. Zero O_2 was determined by injecting a sodium hydrosulfite solution into the sample chamber until no further decrease was observed.

Isolation of heart mitochondria

Mitochondria were isolated from veal heart by a modification of the method of Blair (64). Heart muscle (300 g) was diced and homogenized in 400 ml of 0.25 M sucrose, 0.01 M Tris-Cl, 1 mM Tris succinate, 0.2 mM EDTA, pH 7.8 (sucrose solution) at 4°C. After filtering through a double layer of cheesecloth and adjusting the pH to 7.8 with 2 N KOH, the mince was centrifuged for 15 min at 1200 x g. The supernatant was then centrifuged for 15 min at 26,000 x g. The pellet was resuspended and centrifuged two more times, then resuspended in 10 ml of sucrose solution, divided into fractions and frozen at -40°C. Mitochondrial protein concentration was determined using the BCA protein assay using bovine serum albumin as a standard. The efficiency of the isolation procedure was validated by checking the succinate-dependent membrane potential as an indicator of mitochondrial function and integrity of the inner mitochondrial membrane.

Fluorescence assay of NADH oxidation

Oxidation of NADH was observed by recording the fluorescence using a Perkin Elmer spectrofluorometer set at an emission wavelength of 460 nm and an excitation wavelength of 350 nm. The fluorometer was equipped with a water-jacketed cell holder

that maintained the sample temperature at 37⁰C. The reaction sample included 2 ml of phosphate buffer (pH 7.0) containing heart mitochondria and 1.25 mM KCN. NADH was added to a final concentration of 100 µM. After recording a stable initial fluorescence, NADH oxidation was begun by adding 3-MAQ (50 µM final concentration).

Absorbance assay of superoxide production

Production of superoxide was measured by recording the absorbance increase caused by reduction of partially acetylated cytochrome c. Partially acetylated cytochrome c (Sigma) is reduced by superoxide but, unlike native cytochrome c, it is not a good substrate for mitochondrial enzymes. The assay was performed by recording absorbance at 550 nm on a Shimadzu UV160V spectrophotometer. The reaction mixture included 2 ml of phosphate buffer (pH 7.2) containing heart mitochondria, 1.25 mM KCN, and partially acetylated cytochrome c (100 µg/ml). After recording baseline for 25 sec, NADH was added to a final concentration of 500 µM.

Preparation of HOCD and cysteinyl-dopamine

HOCD was prepared by adding dopamine and cysteine (250 µM and 300 µM final concentrations, respectively) and 100 units of tyrosinase to 4 ml of F12K medium without serum at 37⁰C. After stirring for 15 min, NaOCl was added to 1 mM. The product was sterilized by filtration and used immediately for experiments with PC12 cells. Cysteinyl-dopamine was made in the same way except addition of NaOCl was omitted. Concentrations of these dopamine products are given as the original dopamine concentration.

Measurement of cytotoxicity

Toxicity of redox cyclers was measured using the trypan blue exclusion assay. PC12 cells (ATCC Cat# CRL-1721.1, RRID: CVCL F659) were grown in six-well plates in F12K medium supplemented with 15% heat-inactivated horse serum, 2.5% fetal bovine serum and 1% penicillin/streptomycin/glutamine at 37°C and 5% CO_2. Cysteinyl-dopamine (cysDA) and HOCD were prepared in F12K medium without serum, filter-sterilized and then diluted to the desired concentration with complete medium. Cells at a density of 10^5/ml were treated with cysDA and HOCD. After the desired treatment time, cells were detached using 0.1% trypsin, centrifuged, and resuspended in 0.2% trypan blue in Hanks Buffered Salt Solution (HBSS). After 10 min, cells were placed in a hemocytometer and live and dead cells were counted.

Western blotting

Cells were lysed using triple detergent lysis buffer with protease inhibitor (S8820, Sigma). The lysis buffer included 50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% IGEPAL and 0.05% deoxycholate. Protein concentrations of whole cell lysates were determined using the Bio-Rad DC protein assay reagent in accordance with the manufacturer's protocol. For each cell lysate, 20 µg of protein was mixed in an equal ratio (1:1) with 1X SDS sample buffer and denatured at 95°C for 10 minutes. Denatured samples were then resolved by standard SDS PAGE using 8% polyacrylamide gel. Proteins were transferred (semi-dry) on to PVDF membranes (88518, Thermo Scientific) as described by Canelle et al. (65). Primary antibodies used to probe the blots were: MPO (1:250, ab65871, Abcam), p53 (1:500, ab131442, Abcam), caspase-9 (1:400, ab2325, Abcam), PARP (1:4000, #9542, Cell Signaling) and GAPDH (1:8000, MAB374, Millipore). Corresponding HRP-conjugated secondary antibodies used were as follows: goat anti-rabbit IgG

(ab6721, Abcam) and sheep anti-mouse IgG (NA931, GE Healthcare). The antibody signals were detected using the ECL Prime Western Blotting detection agent (Amersham, GE Healthcare). Blots were photographed using the FOTO/Analyst Investigator (FOTODYNE) and quantified using the TotalLab TL 100 software (Nonlinear Dynamics). Quantified values of the test protein were normalized to the loading control, GAPDH.

Myeloperoxidase chlorination activity assay

Chlorination activity of myeloperoxidase in whole cell lysates was determined using the EnzChek Myeloperoxidase (MPO) Activity Assay Kit (E33856, ThermoFisher Scientific). The assay was performed as per the manufacturer's protocol. Chlorination activity was measured as fluorescein yielded by the selective cleavage of non-fluorescent 3'-(p-aminophenyl) fluorescein (APF) by hypochlorite. The fluorescence intensity was measured with excitation at 485 nm and emission at 530 nm, using a SpectraMax Gemini XPS plate reader (Molecular Devices).

Observation of superoxide production using MitoSOX

PC12 cells were incubated in Hanks Buffered Salt Solution (HBSS) containing MitoSOX Red^{TM} (2.5 µM final concentration) for 10 min at 37ºC. The buffer with MitoSOX dye was aspirated, cells were washed twice with fresh HBSS and placed in an Olympus IX81 ZDC inverted microscope equipped with a custom-built incubator for live-cell imaging. Bright-field and fluorescence images (rhodamine filter set) were acquired at 37°C. Redox cycler in 1 ml of HBSS prewarmed to 37ºC was added to initiate superoxide generation. After 5-10 minutes, another fluorescence image was obtained. A 10×/0.25 NA CP-Achromat lens and a 40×/0.75 NA Plan-Neofluar lens were used for phase contrast images and fluorescence images respectively. All images were acquired with a

Diagnostic Instruments (MI, USA) Boost EM-CCD-BT2000 camera driven by IPLab software (BD Biosciences, MD, USA). Images were optimized using ImageJ software.

Statistical analysis

An unpaired t-test was used to calculate p values. For toxicity, 9 samples in each group were compared (16 degrees of freedom). Myeloperoxidase activities were compared with 4 samples in each group (6 degrees of freedom) and myeloperoxidase expression with 3 (4 degrees of freedom). Significance is indicated as ns ($p \geq 0.05$), *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), ****($p < 0.0001$).

Materials

Myeloperoxidase (EMD Millipore) was dissolved in pH 6 phosphate buffer at a concentration of 0.33 mg/ml. Sodium hypochlorite (140 mM) was prepared by mixing 0.375 g of calcium hypochlorite with 1.0 g of sodium carbonate in 25 ml of H_2O and removing the precipitate by filtration. Hypochlorite was assayed by the method of Han et al. (66). 3-Methyl-5-anilino-o-quinone (3MAQ) was synthesized by oxidizing 3-methylcatechol in the presence of aniline (67). Mushroom tyrosinase, bovine liver catalase, superoxide dismutase, menadione, 6-hydroxydopamine and 9,10-phenanthrenequinone were purchased from Sigma/Aldrich and MitoSOX Red from Life Technologies.

CHAPTER 3 – REDOX CYCLING

INTRODUCTION

Oxidation/reduction (redox) reactions, as the name suggests, involve both reduction and oxidation. They are chemical reactions in which one or more electrons are transferred between two compounds. This electron transfers either results in an increased oxidation state (oxidation) of a molecule due to the loss of electrons or a decreased oxidation state (reduction) of a molecule due to the gain of electrons. The oxidized and reduced forms of the same molecule are together called a redox couple. Redox reactions typically occur in pairs, one redox couple being reduced and the other being oxidized.

The tendency of a molecule to either donate or accept electrons reflects its reduction potential. The standard reduction potential (E_0) of a molecule is determined by comparing its value to that of hydrogen, which is arbitrarily set at zero. Thus, strong oxidizing agents have positive redox potentials whereas reducing agents have negative redox potentials. The difference in reduction potential between two molecules will determine the equilibrium between their oxidized and reduced states. Redox couples with lower reduction potentials will donate electrons to reduce redox couples with higher potentials. When many different redox active intermediates are present, redox reactions often occur as a chain of successive redox reactions.

Regulating redox activities is imperative for the control of several cellular signaling pathways. Thus, in order to define different processes in redox biology, it is not only necessary to understand the chemistry underlying the redox reactions but also appreciate the biological context that supports these reactions. Factors such as concentration, pressure, pH and temperature affect the redox potential of a molecule.

There are many redox couples that contribute towards maintaining the redox environment, a prominent one being the abundantly found GSSG/2GSH couple (glutathione). The half-cell reduction potential (E_{hc}) of the GSSG/2GSH couple varies according to the biological status of the cell; during proliferation $E_{hc} \sim$ -240 mV; during differentiation $E_{hc} \sim$ -200 mV; or during apoptosis $E_{hc} \sim$ - 170 mV (68). These potentials are useful in deducing the redox status and associated oxidative stress in a cell. Using this approach of quantitative biology thereby offers a rationale to explore the cellular mechanisms involved with cell growth and development, signaling, oxidative stress and/or apoptosis.

Reduction potential is an important parameter for reactions involving redox active metabolites. However, under physiological conditions, redox couples are rarely in equilibrium. The concentrations of reduced and oxidized forms are subject to constant variation due to fluctuating enzyme concentrations and activities, as the result of metabolism. The degree of redox activity in a cellular environment, at any point of time, depends on the physiological condition in conjunction with the number and state of the redox active moieties involved.

Redox cycling involves continuously coupled reduction and oxidation reactions, often involving oxygen and reactive oxygen species. In theory, any compound with the tendency to accept or donate electrons can participate in redox cycling. Redox reactions are reversible and most redox active compounds can be either oxidants or reductants depending on the biological conditions. Redox-cycling occurs when a compound undergoes alternating reduction and oxidation reactions.

In the first step of redox cycling, the redox cycler undergoes reduction in the presence of a reducing agent. Re-oxidation of the reduced form takes place most significantly in the presence of molecular oxygen. The intermediate donates a single electron to oxygen thereby reducing it to the superoxide radical anion. This will result in the regeneration of the original parent compound in addition to the superoxide anion. The reduction of O_2 to superoxide is especially significant because it occurs spontaneously (non-enzymatically) by outer-sphere electron transfer and it yields the highly reactive superoxide. Superoxide generation further leads to the formation of hydrogen peroxide either spontaneously or by the catalytic action of superoxide dismutase. If hydrogen peroxide encounters transition metals under physiological milieu, it can be converted to highly toxic and potentially damaging hydroxyl radical and singlet oxygen (69,70). Thus, redox cycling leads to the proliferation of a variety of reactive oxygen species.

Redox cycling compounds, based on their redox potential, can accept electrons from biologically available reducing agents such as reduced flavoproteins, NADPH, NADH, reduced ferredoxin, reduced glutathione (GSH) and other thiol containing compounds and ascorbic acid. Under physiological conditions, some enzymes are also known to catalyze such reactions. These include NADPH-cytochrome P-450 reductase, xanthine oxidase, mitochondrial flavoproteins of the electron transport chain and many other dehydrogenases (69,70).

The process of redox cycling involving these alternating reduction and oxidation reactions continues until the molecular oxygen and/or reducing equivalents are exhausted. This process is sometimes also referred to as "futile cycling." This process of redox cycling could eventually lead to cytotoxicity via depletion of essential reducing

equivalents, depletion of oxygen or via potential interaction between free radicals and critical biological macromolecules (69,70). Radicals released during redox cycling can by themselves engage in free radical-mediated toxic reactions. They have the tendency to bind covalently to macromolecules and cause lipid peroxidation, DNA damage or protein inactivation. Moreover, interaction of free radicals with smaller biomolecules such as glutathione could be equally damaging. Glutathione is not only an integral cellular defense against cytotoxic agents but also plays a major role in other biological functions such as deoxyribonucleotide synthesis. During redox cycling, glutathione tends to compete with molecular oxygen for anion radicals, which leads to depletion of the reduced form of glutathione. Based on the degree of release of free radicals, it could also lead to disorganization of membrane structure and ultimately disruption of cytoskeleton and cell death.

Under conditions of relatively high molecular oxygen, toxicity could result due to the generation of hydroxyl radicals or from the depletion of important reducing agents. On the contrary, under low oxygen levels, induction of a hypoxic state and/or an increase in the organic radical levels are the predominant causes of toxicity (69). From an experimental perspective, redox cycling and its subsequent ROS generation tend to interfere with enzymes or reagents in biochemical assays and high throughput screens giving false positive results (71-73).

Redox cycling of quinones has been a well-studied phenomenon and its connection to toxicity has been firmly established (74). Quinones have a tendency to undergo one- or two-electron reduction reactions. In the case of the former, radical semiquinones are formed and due to their highly unstable nature, one-electron reduction

generates reactive species (69,70). Two-electron reduction on the other hand, mostly catalyzed by diaphorases, results in the formation of completely reduced and biologically inactive hydroquinones. This is usually considered as a detoxification process (75,76). Supporting this theory, Thor et al. have shown that inhibiting DT diaphorases enhances the toxicity of quinones by leaving them free to undergo one-electron reduction to semiquinones (76). Numerous redox cycling compounds have been reported to have deleterious biological effects due to their conversion to quinones and semiquinones. Apart from the quinones, there are a number of naturally occurring benzophenanthridine compounds such as sanguinarine and chelerythrine, that also redox cycle generating free radicals and leading eventually to apoptosis (77). Though a number of studies have focused on the link between redox cycling and toxicity, very few studies have considered the relation between the mode of redox cycling and toxicity.

In the case of Parkinson's disease, one plausible mechanism that links dopamine oxidation to oxidative stress, and which possibly accounts for selective loss of dopaminergic neurons, is the redox biochemistry specific to dopamine. Multiple pathways of dopamine oxidation have been identified. At physiological pH, dopamine can auto-oxidize forming reactive oxygen species which can damage cellular constituents such as lipids, proteins and DNA. Auto-oxidation of dopamine can lead to the formation of dopamine-quinones that can easily react with cellular nucleophiles. Both *in vitro* and *in vivo* studies have shown dopamine-derived quinones to react with thiols of proteins. Within striatal nerve terminals, the concentration of dopamine has been estimated to be around 50 mM (78). Reports have demonstrated that increased dopamine oxidation in the presynaptic cleft leads to an alteration in the redox status of dopamine terminals (79).

With specific reference to DA being considered as an endogenous neurotoxin, two independent studies have established that DA quinones inactivate tyrosine hydroxylase by covalently modifying its sulfhydryl groups. This converts the enzyme to a redox-cycling quino-protein which can react with transition metals via Fenton chemistry and thereby cause oxidative stress and consequent dopamine synthesis failure. Compounds with low potentials are known to undergo redox cycling, e.g., quinones such as menadione and catecholamine derivatives such as 6-hydroxydopamine, adrenochrome and aminochrome. Redox cycling of compounds like aminochrome and adrenochrome have been suggested to cause the death of dopaminergic cells in Parkinson's disease. However, these catecholamine derivatives have a tendency to oxidize to higher potential dihydroxyindoles, making them highly unstable and difficult to study their biological effects and redox cycling mechanism.

Nonetheless, in regards to Parkinson's disease it is important to consider that mild oxidative stress persists for years in nigrostriatal neurons, which could ultimately evoke a biological condition leading to untimely death of the dopamine neurons. Evidence in support of this has also shown traces, at autopsy, of oxidized dopamine metabolites in nigral neurons (63,80-82).

Studies with biological systems have shown increased generation of hydrogen peroxide with cell-specific redox cycling of toxic products such as paraquat, doxorubicin and alloxan (83). This has led to cellular destruction and subsequent phagocytosis. Similarly, in brain, redox cyclers like, 6-hydroxydopamine and 6-aminodopamine have been shown to specifically damage catecholamine neurons (84). Also, in addition to hydrogen peroxide, other species including hypochlorous acid, reactive quinones,

hydroxyl radicals and superoxides have also been shown to contribute to the stress in dopamine nerve terminals.

Redox cycling compounds are prooxidant catalysts that transfer electrons to oxygen to generate reactive oxygen species (75,76). Commercially, such compounds are used as substituents in xenobiotic compounds (e.g. redox active pesticides) (85-89), redox active pharmacophores (e.g. anesthetics) (90) and in pharmaco chemotherapeutic drugs (e.g. menadione, doxorubicin/adriamycin) (91-94). Studies have shown that an inability to detoxify the redox cycling agents has led to increased vulnerability to environmental hazards. From a pharmaceutical perspective, this has also introduced some limitations in anticancer chemotherapeutics and antibiotics. Since there may be an age-related decline in the capacity to detoxify redox cycling compounds, it is extremely important to have detailed toxicity profiles of such redox cyclers from a therapeutic point of view.

Therefore, the aim of this study is to understand the different modes of redox cycling by comparing chemically synthesized redox cyclers.

RESULTS

Over the years, consumption of oxygen and production of superoxide anions have been used for measuring redox cycling activities. For our experimental purposes, we employed a convenient assay to study redox cycling, where we measure oxygen consumption using a Clark-type oxygen electrode. The rate of oxygen consumption is directly proportional to the redox cycling activity of the compound studied. The compounds used in our work were 9,10-phenanthrenequinone, menadione, 6-hydroxy dopamine and 3-methyl-5-anilino-1,2-benzoquinone. Among these, 3-MAQ is a

chemically synthesized, laboratory developed redox cycler that structurally mimics and shares some electrochemical properties with the oxidized dopamine metabolite aminochrome. The others are commercially available chemically synthesized compounds.

To study the redox cycling process, ascorbic acid and dithiothreitol (DTT) were used as reducing agents. Both ascorbate and DTT were used to catalyze non-enzymatic reduction of the redox cyclers. As observed in fig. 2, all four compounds did undergo ascorbate- as well as DTT-dependent redox cycling.

When ascorbic acid is used as an electron donor, one-electron reduction of the redox cycler takes place (Fig. 1). As a result, the quinone is reduced to a semiquinone radical and ascorbate is oxidized to semidehydroascorbate. The semiquinone reacts with O_2 yielding superoxide and the oxidized quinone. The superoxide produced in this scenario reacts with ascorbate as well as semidehydroascorbate. Thus, when superoxide dismutase is added alongside ascorbic acid, there is no effect on the redox cycling activity.

DTT is a strong reducing agent that usually catalyzes two-electron reduction reactions. It tends to reduce the quinone redox cycler by donating two electrons to form a hydroquinone (Fig. 1). The relatively stable hydroquinone does not react readily with O_2, so it must be oxidized by superoxide. The semiquinone formed in this first step is then oxidized back to its parent quinone by molecular oxygen. This second step generates the

$$QH_2 \quad \xrightarrow[\quad]{O_2^{\bullet-} \quad H_2O_2} \quad Q^{\bullet-} \quad \xrightarrow[\quad]{O_2 \quad O_2^{\bullet-}} \quad Q$$

Ascorbic Acid
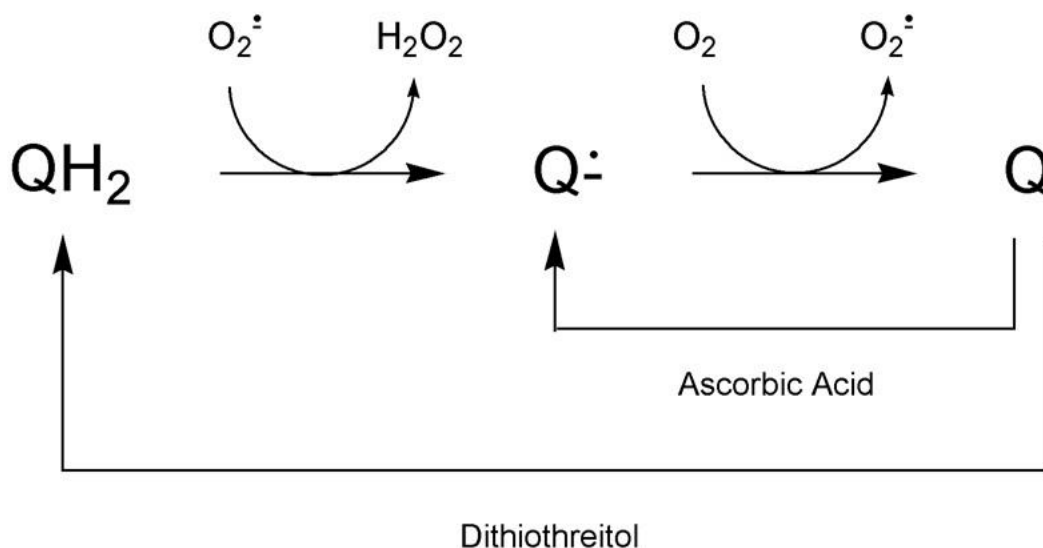
Dithiothreitol

Figure 1. One-electron vs. two-electron mediated redox cycling – Ascorbic acid mediates one-electron reduction forming the semi-quinone (Q-); dithiothreitol mediates two-electron reduction forming the fully reduced hydroquinone (QH_2). Whereas O_2 oxidizes the reactive semiquinone, superoxide is required to oxidize the hydroquinone.

superoxide anions needed for the first step. Thus, when superoxide dismutase is added

to the solution along with DTT, redox cycling is inhibited. This is because superoxide

dismutase eliminates superoxide anions from the solution, inhibiting the conversion of

hydroquinone to the semiquinone anion. Because some superoxide is undoubtedly lost

by disproportionation and reaction with DTT, there must be other mechanisms for

converting the corresponding fraction of hydroquinone to the semiquinone. Otherwise,

the hydroquinone would accumulate and redox cycling would stop. There could be a

comproportionation reaction in which the hydroquinone and quinone forms of the redox

cycler react to form the semiquinone radical anion. The hydroquinone might also react

slowly with O_2, especially in the case of low-potential redox cyclers.
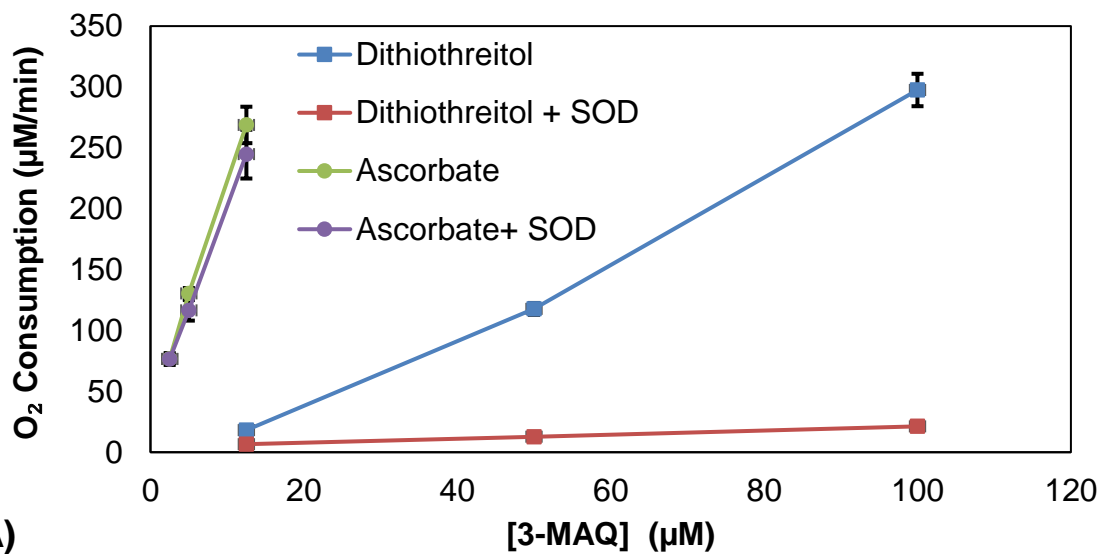
A)
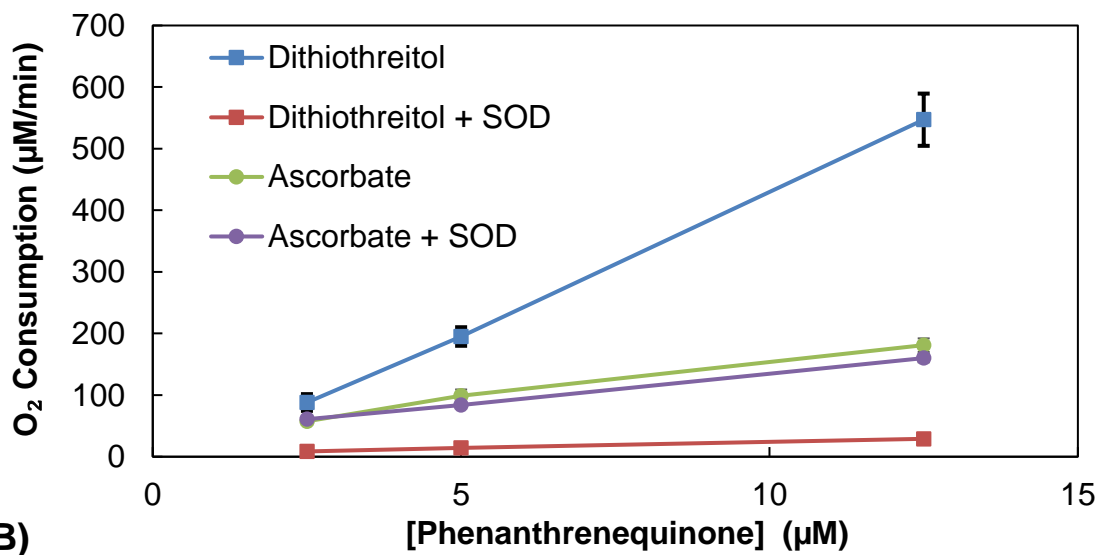
B)
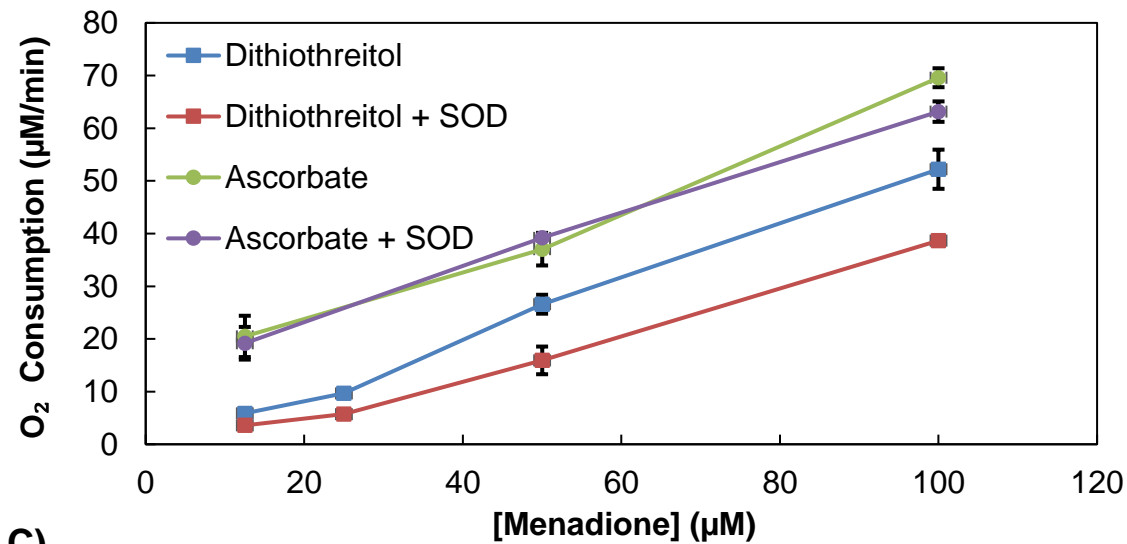
C)
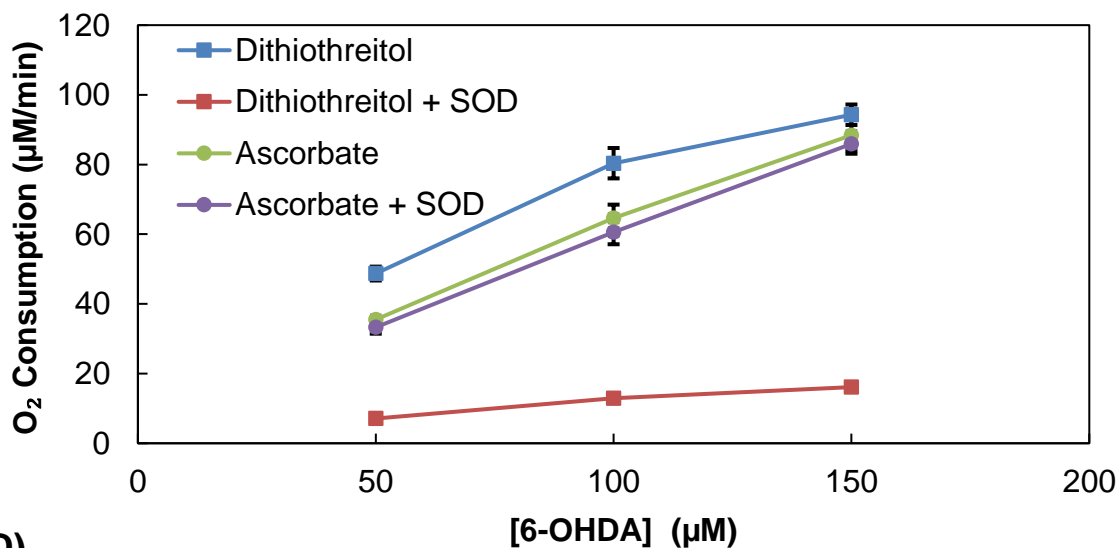
D)

Figure 2. Inhibition of ascorbate and dithiothreitol-driven redox cycling by superoxide dismutase – Redox cycling was monitored as O_2 consumption at 37 °C and pH 7.4, in a Clark-type oxygen electrode. The redox cycling activity of varying concentrations of (A) 3-MAQ; (B) 9,10-Phenanthrenequinone; (C) Menadione; and (D) 6-hydroxydopamine, was initiated by adding 2.5 mM ascorbic acid (green) or 250 µM dithiothreitol (blue). Superoxide dismutase when added; inhibited the redox cycling activity with dithiothreitol (red) as the electron donor but had no effect with ascorbate (purple) as the electron donor.

Of the four redox cyclers tested, it is apparent that DTT-driven redox cycling of menadione is less sensitive to superoxide dismutase. This may be because menadione comproportionates faster than the other compounds or that its hydroquinone reacts more readily with O_2. Menadione does have the lowest reduction potential of the redox cyclers tested (-5 mV compared to +10 mV to +90 mV for the others).

6-Hydroxydopamine (6-OHDA) is an endogenous neurotoxin formed by dopamine oxidation, and it is commonly used as a dopaminergic neurotoxin to mimic the effects of Parkinson's disease. It was tested here to characterize the redox cycling mechanism of this oxidized dopamine metabolite. 6-Hydroxydopamine is highly unstable in aqueous solution, and it spontaneously reacts with molecular oxygen changing a colorless solution to bright red, with a maximum absorbance at 490 nm. This is due to the formation of the quinone form of 6-OHDA. Interestingly, addition of DTT immediately changes the solution to colorless, suggesting rapid reduction of the quinone back to the fully reduced 6-OHDA in a two-electron reduction. In the case of ascorbate, this change is gradual. This suggests that ascorbate reduces the 6-OHDA quinone to the semiquinone in a one-electron reduction. The semiquinone then rapidly reoxidizes by reacting with O_2.

While ascorbic acid is a good reducing agent for redox cycling *in vitro*, it is also a good antioxidant so oxidative stress will be minimal in its presence. Therefore, it is imperative to find another natural cellular reducing mechanism that will permit oxidative damage. Enzymatic reduction is likely, and the mitochondria are of particular interest because of their high and continuous production of reduced electron carriers (i.e. NADH and $FADH_2$).

Mitochondria-driven redox cycling of 3-MAQ was checked using a Clark-type oxygen electrode (Fig. 3). Potassium cyanide was added to eliminate any interfering O_2 consumption catalyzed by the respiratory chain in the mitochondria. Typically, this interference was small, however, as eliminating KCN from the reaction sample yielded similar results. After 5 minutes, either 40 µl of 10 mM 3-MAQ or 40 µl of 50 mM reduced nicotinamide adenine dinucleotide (NADH) was added, followed 5 minutes later by the addition of the other. The redox cycling activity of 3-MAQ was measured as the rate of oxygen consumption. Addition of only 3-MAQ did not show any redox cycling activity indicating the absence of any endogenous NADH. Also, eliminating mitochondria diminished the redox activity. This confirmed the need for both mitochondria and NADH in the redox cycling of 3-MAQ
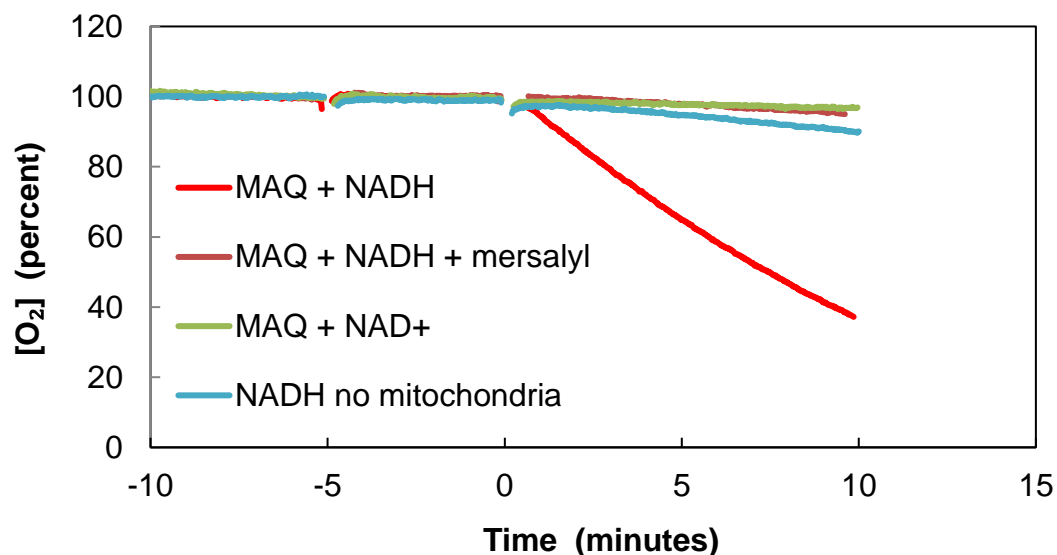
Figure 3. NADH-dependent redox cycling in veal mitochondria – The reaction mixture containing 4 ml of phosphate buffer (pH 7.0), 100 µl of veal brain mitochondria and 50 µl of 100 mM potassium cyanide (KCN) was placed in the oxygen electrode chamber at 37°C. 3-MAQ (100 µM final concentration) was added at time = -5 min and 50 mM NADH (500 µM final concentration) was added at time = 0. The redox cycling activity of 3-MAQ was observed as the consumption of oxygen. It was not observed in the presence of 50 µl of 50 mM NAD+ or 40 µl of 250 mM mersalyl acid, or in the absence of mitochondria.

Once the redox cycling of 3-MAQ was found to be NADH-dependent, we confirmed

the oxidation of NADH by looking at the NADH fluorescence during redox cycling (Fig. 4).

Fluorescence of NADH was recorded using a Perkin Elmer spectrofluorometer set at an

emission wavelength of 460 nm and an excitation wavelength of 350 nm. Addition of 10

µl of 10 mM 3-MAQ, oxidized the NADH, as seen by the linear decrease in fluorescence.

The immediate drop in fluorescence following 3-MAQ addition is an artifact caused by the

absorbance of 3-MAQ.
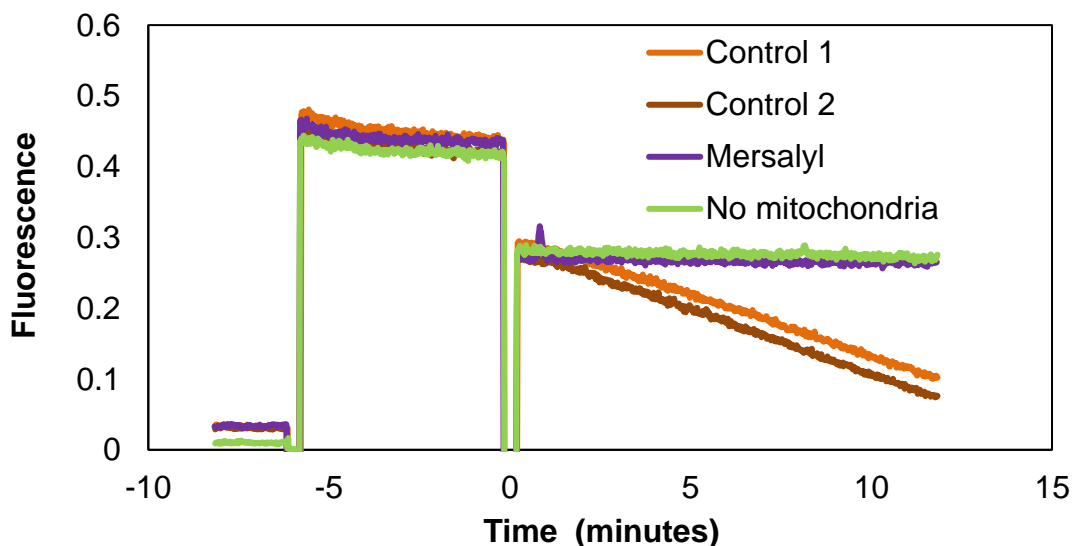
Figure 4. Oxidation of NADH during redox cycling of 3-MAQ – NADH was measured
by fluorescence. The reaction sample included 2 ml of phosphate buffer (pH 7.0), 20 µl
heart mitochondria and 25 µl of 100 mM KCN. 40 µl of 5 mM NADH was added at -6 min.
Addition of 10 µl of 10 mM 3-MAQ at time = 0 initiated oxidation of NADH confirmed by
the drop in the fluorescence readings. NADH oxidation was inhibited in the presence of
20 µl of 250 mM mersalyl acid and when mitochondria were omitted.

Inhibitors of various mitochondrial functions including the respiratory chain (rotenone, cyanide, quercetin, antimycin), xanthine oxidase (allopurinol) and NADH:quinone oxidoreductase (dicumarol) were tested for inhibition of the redox cycling activity of 3-MAQ (data not shown). None, except mersalyl acid, inhibited NADH-dependent redox cycling (Fig. 3). Mersalyl acid seemed to inhibit the redox cycling activity of 3-MAQ almost completely. Mersalyl acid is known to inhibit cytochrome b5 reductase. This hints at the possibility that cytochrome b5 reductase could be the primary mitochondrial enzyme that catalyzes the redox cycling of oxidized dopamine products (3-MAQ in this case). To test this possibility, we examined the effect of cytochrome b5 on 3-MAQ redox cycling in the oxygen electrode. Human recombinant cytochrome b5 (20 µg/ml final concentration) was added to the oxygen electrode in 4 ml of 0.2 M phosphate buffer (pH 7.0) containing heart mitochondria, cyanide and 3-MAQ (100 µM final concentration). Redox cycling was initiated by adding NADH (0.5 mM final concentration).

Presence of cytochrome b5 increased the redox cycling activity of 3-MAQ, as indicated by a noteworthy increase in the rate of oxygen consumption (Fig. 5). Although there is an apparent increase in 3-MAQ redox cycling due to cytochrome b5, what role it plays in this mechanism and how it interacts with cytochrome b5 reductase in this experiment is still not clear.

Of special interest is whether NADH-driven redox cycling of 3-MAQ produces superoxide in the presence of mitochondria. This was checked by assaying superoxide using acetylated cytochrome c (95). Acetylated cytochrome c is readily reduced by superoxide but is relatively less susceptible to direct reduction by mitochondrial enzymes than the native protein, thereby providing less interference from competing reactions.
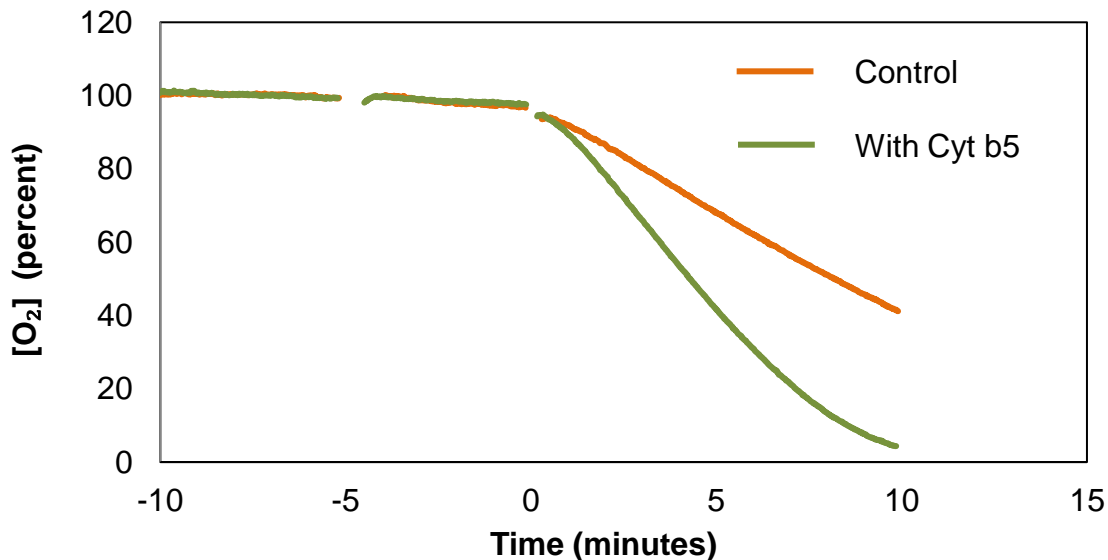
Figure 5. Effect of cytochrome b5 on NADH-dependent redox cycling – Redox cycling of 3-MAQ (100 μM final concentration) was tested in the oxygen electrode in the presence and absence of 40 μl of human recombinant cytochrome b5 (1mg/ml); NADH (500 μM final concentration) was also added at time=0. The presence of cytochrome b5 caused a noteworthy increase in the rate of oxygen consumption.
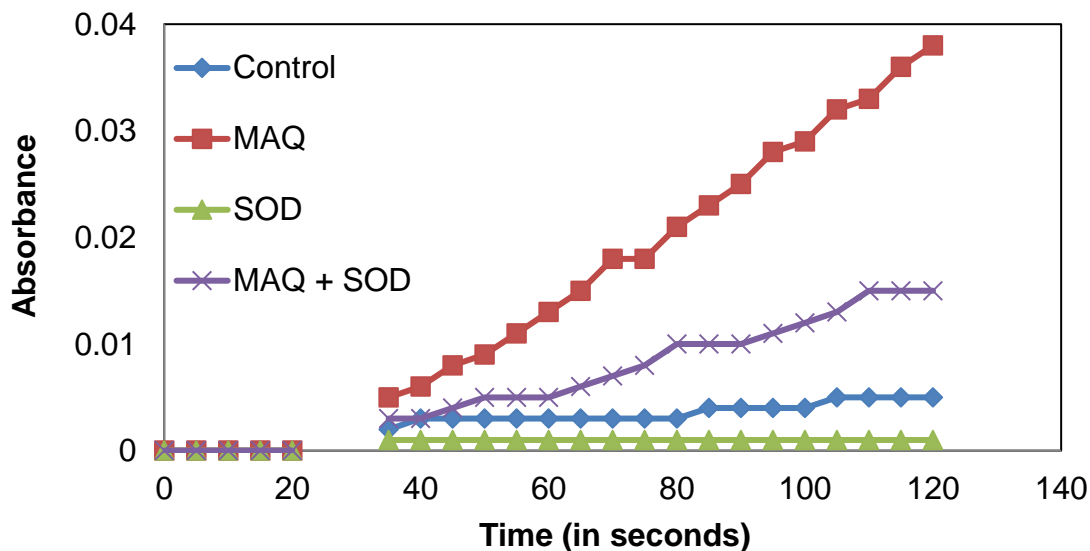
Figure 6. Superoxide production observed by cytochrome c reduction – Cytochrome c reduction was recorded at 550 nm. The reaction mixture including 2 ml of mitochondrial assay buffer (pH 7.2), 10 μl of veal heart mitochondria, 25 μl of 100 mM KCN and 20 μl of acetylated cytochrome c was placed in a cuvette and absorbance was recorded for 2 minutes. 20 μl of 50 mM NADH was added at time = 25 sec. 10 μl of 2.5 mM 3-MAQ when added, caused the production of superoxide anions, whereas presence of 20 μl of 3000 U/ml of superoxide dismutase decreased the production of superoxide anions.

The assay was performed by recording the cytochrome c reduction as the increase in absorbance at 550 nm. As predicted, superoxide production was observed with acetylated cytochrome c, as detected by the decrease in absorbance (less cytochrome c reduction) when superoxide dismutase was present (Fig. 6).

DISCUSSION

It is generally believed that one-electron reduction generates reactive oxygen species whereas two-electron reduction leads to detoxification (69,70). One-electron reduction reactions are mediated by a number of reductive enzymes, namely, microsomal NADPH-cytochrome P450 reductase and mitochondrial NADH-ubiquinone oxidoreductase (complex I) (96). The unstable semi-quinone formed in the process can readily be oxidized by molecular oxygen, resulting in reformation of quinone and generation of reactive oxygen species. In contrast, two-electron reduction of quinones is catalyzed by reductive enzymes, such as NAD(P)H:quinone oxidoreductase 1 (EC 1.6.99.2, NQO1; DT-diaphorase), where the resulting hydroquinone is comparatively more stable than the semi-quinone (97). Thus generation of ROS is greatly diminished.

These two cases are analogous to the two-electron dithiothreitol- and one-electron ascorbic acid-dependent redox cycling mechanisms respectively. Can general principles derived from *in vitro* studies of redox cycling be applied to understand observations *in vivo*? The inhibition of dithiothreitol-dependent redox cycling by superoxide dismutase implies that two-electron reduction of quinones is protective only as long as superoxide concentrations are kept low. If superoxide increases, it will oxidize the reduced quinones to their more reactive semiquinones.

Among the redox cyclers that we studied, menadione is an interesting special case. It has been previously shown to induce lipid peroxidation and membrane damage and to decrease reduced glutathione levels due to its redox cycling capability (76,98-101). Criddle et al. (2006) have shown that redox cycling of menadione generates reactive oxygen species and leads to apoptotic cell death of pancreatic acinar cells (97). They also showed that redox cycling of menadione led to a concomitant decrease of NAD(P)H, thereby hindering the activity of two-electron detoxifying enzymes such as NAD(P)H:quinone oxidoreductase. In my *in vitro* studies, menadione is actually a relatively slow redox cycler compared to the other compounds. However, its DTT-dependent redox cycling is also relatively insensitive to superoxide dismutase. Thus, menadione may be unexpectedly effective *in vivo* because it can redox cycle in the presence of two-electron quinone reduction and low superoxide levels.

Redox cycling quinones have been implicated in many forms of neurotoxicity (102,103). When a quinone undergoes one-electron reduction, a semiquinone is generated. This semiquinone gets converted back to its parent quinone form by donating one electron to dioxygen (104). Redox cycling of quinones is tightly restricted. If a quinone is a strong oxidant then semiquinone formation is favored, but not the regeneration of the parent compound. If a quinone is a strong reductant, then reaction of the semiquinone with oxygen is favored, but not the formation of the semiquinone. A good redox cycler, therefore, is ideally a compound that can be easily oxidized and can then be reduced back to its original form. They typically have reduction potentials in the range of -100 mV to +100 mV. Some of the organic chemical compounds capable of undergoing one-electron reduction and redox cycling are azo derivatives, nitroso compounds, N-oxides,

S-oxides and polyhalogenated aliphatic hydrocarbons. Contrarily, carbonyl compounds do not favor generation of radical intermediates, because they undergo dehydrogenase-catalyzed two-electron mediated reduction reactions. Physiological conditions are ideal for the reduction and redox cycling of these compounds, since live cells have strongly negative reduction potentials (69,70).

Quinones are electron-deficient compounds which are highly reactive with nucleophilic groups such as thiols and protein sulfhydryls (105). *In vivo*, therefore, quinones can react with and covalently link to proteins. Antioxidants and reductants can protect against quinone-induced damage by competing with protein sulfhydryls for the quinones (61,106-108). Interestingly, the rate of redox cycling can be more pronounced when the quinone is bound to a protein rather than in its free form in solution. In support of this conclusion, the effects of quinoproteins have been found to last longer than those of reactive oxygen species or quinone by itself, in solution (61,106,109). Also, antioxidants, that usually serve as cellular defense mechanisms protecting against oxidative stress, have been reported to contribute to redox cycling of a protein bound quinone, which also depletes cellular energy and endogenous reducing equivalents (103,110,111).

Some compounds have been proposed to redox cycle *in vivo* although they do not redox cycle *in vitro*. In the MPTP model for Parkinson's disease, MPTP is converted to its more toxic compound MPP+. Neither MPTP nor its oxidized counterpart can redox cycle. MPTP itself is highly stable under normal conditions and does not autoxidize. When MPTP undergoes two-electron as well as four-electron oxidation, neither of the oxidation products of MPTP can be reduced easily (112). Chacon et al. (1987) showed that the

reduction potential of MPP+ (-1V) at physiological pH makes it a poor redox cycling agent (113). Nevertheless, it has still been suggested that MPP+ gets reduced to the MPP radical, which undergoes redox cycling (114). Whether MPP+ leads to oxidative stress via redox cycling is still an unanswered question.

Paraquat, a well-known herbicide, is structurally similar to MPP+ and has been widely used to study Parkinson's disease. Animal models chronically treated with paraquat have demonstrated loss of dopaminergic neurons in the substantia nigra. The toxicity of paraquat has been attributed to its ability to redox cycle (115). It has a reduction potential of -0.450 V, which is significantly higher than that of MPP+ but still very low for a redox cycler. Some studies have questioned the redox cycling capability of paraquat. In an assay for redox cycling, paraquat did not produce hydrogen peroxide and therefore, levels of reactive oxygen species were low (116).

How compounds that fail to redox cycle *in vitro* can appear to do so *in vivo* is therefore a mystery. Whether they metabolize to other redox cycling compounds, couple to proteins to form redox cycling adducts, or inhibit activities that allow the redox cycling of endogenous compounds are all possibilities. There are numerable factors that determine the amount of biologically active, reactive intermediates formed by redox cycling. Among the two major factors involved, one is the redox potential of the compound to be reduced and another is the availability and activity of the enzymes catalyzing the reduction reactions. The energy status as well as the oxygen tension of the affected cell population also impact the redox cycling process (69,70). Another factor that influences the equilibrium reaction of a redox cycler with oxygen is superoxide dismutase. Superoxide dismutase catalyzes dismutation of superoxide to hydrogen peroxide and

water further driving the reaction towards radical formation. Other variables that may affect the redox cycling mechanism under certain conditions are the availability of reducing equivalents and cellular concentrations of antioxidants (69,70).

Although redox cycling has been a well-studied phenomenon *in vitro*, inconsistent observations *in vivo* have remained a persistent challenge for the research community. It is important to consider the translational gap that exists between *in vitro* and *in vivo* studies. A compound capable of redox cycling and producing free radicals *in vitro*, may not follow suit *in vivo* (117). As a first step towards narrowing this gap, it is necessary to demonstrate the generation of free radicals *in vivo* (117). A thorough understanding of the structural and physicochemical aspects of redox cycling is required. This would further help in generating compounds, capable of influencing cellular redox status, with therapeutic applications.

CHAPTER 4 – DOPAMINE, HYPOCHLORITE AND PARKINSON'S DISEASE

INTRODUCTION

The dopamine oxidation pathway has been studied extensively. Dopamine is usually sequestered in to intracellular vesicles that prevent it from oxidizing under the physiological conditions of the cytosol. In PD patients, it has been reported that the activities of vesicular monoamine transporter 2 (VMAT2) (118) and the cell membrane dopamine transporter (DAT) (119) are diminished, so the possibility of free dopamine in cytosol/synapses increases considerably. Free dopamine that has escaped or been released from this vesicular storage, whether in the cytoplasm of the cell or in the synapse, is more likely to undergo either enzymatic or non-enzymatic oxidative metabolism to deleterious species that exert cytotoxicity through a variety of established mechanisms (53,57). Among dopamine metabolites, 3,4-dihydroxyphenylacetaldehyde (DOPAL), aminochrome, 5-S-cysteinyl dopamine and its metabolite DHBT-1 have all received considerable attention.

Enzymatically, dopamine is metabolized oxidatively by monoamine oxidase (MAO) and catechol-o-methyltransferase. MAO-B, an outer mitochondrial membrane enzyme, catalyzes oxidative deamination of dopamine to 3,4-dihydroxyphenylacetaldehyde (DOPAL) which is more toxic than the parent dopamine (120). DOPAL itself can be converted to a comparatively non-toxic compound DOPAC by aldehyde dehydrogenase (ALDH). In Parkinson's patients, DOPAL is found to be selectively toxic to dopaminergic neurons while ALDH activity is reduced (120). Interestingly monoamine oxidase inhibitors, which would be expected to block the formation of DOPAL, do not appear to slow the progression of Parkinson's disease. Also, within astrocytes, dopamine can be

metabolized into homovanillic acid (HVA) through the synergistic action of catechol-o-methyltransferase (COMT), monoamine oxidase (MAO) and aldehyde dehydrogenase (ALDH) (121,122). Dopamine may also undergo oxidation via minor enzymatic pathways, involving the enzymes prostaglandin H synthase, lipoxygenase, and tyrosinase (123).

Our study, however, focuses on the non-enzymatic oxidation of dopamine and its auto-oxidation metabolites. Dopamine can undergo non-enzymatic spontaneous auto-oxidation to radicals and quinones, with concomitant release of reactive oxygen species (53-56). To begin the non-enzymatic oxidation, dopamine auto-oxidizes slowly in the presence of O_2 to form the o-quinone. The o-quinone is susceptible to nucleophilic attack, and reacts rapidly with thiols such as cysteine, to form adducts such as 5-S-cysteinyl-dopamine (55,61).

Since cysteine residues play an important role in protein function, their modification via the irreversible covalent reaction between dopamine quinone and cysteine can have adverse effects on cellular health (60). Moreover, the basal level of oxidative stress rises in the neurons, due to the formation of the conjugate quinone species, especially as 5-S-cysteinyl species are more easily oxidized than the parent catecholamine. Notably, traces of 5-S-cysteinyl-dopamine have been detected in the cerebrospinal fluid of PD patients, dopamine-rich regions of the brain such as the caudate nucleus, putamen, globus pallidus and substantia nigra, and in neuromelanin (81,124).

Cysteinyl-dopamine has been reported to kill neuronal cells (82), but it is uncertain whether it is cytotoxic itself or metabolizes to toxic products. Cysteinyl-dopamine oxidizes relatively slowly like dopamine itself. In an effort to identify products that react more quickly with O_2 and therefore would generate reactive oxygen species (ROS), we have

sought dopamine metabolites capable of rapid redox cycling. We report here that hypochlorite converts cysteinyl-dopamine into a cytotoxic redox cycling product capable of generating free radicals and thereby creating oxidative stress.

RESULTS

Over the years, a number of studies have shown that cysteinyl-dopamine (cysDA) is toxic to a variety of neuronal cells and cell lines in culture (82,125-127). Here, we found that cysteinyl-dopamine at concentrations of 100 μM or greater is toxic to catecholaminergic PC12 cells (Fig. 8). When cysDA is oxidized with hypochlorite, PC12 cells are more susceptible to the resulting product at lower concentration; the same concentration of hypochlorite by itself is not toxic. Interestingly, addition of taurine to the cells nearly abolishes the toxicity of cysDA for up to 48 hours but has no effect on the toxicity of the hypochlorite-treated product. Taurine is known to be a hypochlorite scavenger (128), suggesting that the toxicity of cysteinyl-dopamine may depend upon its conversion to the hypochlorite product. This product is suspected to be a benzothiazine derivative (see discussion), but as the structure is not yet established, we will refer to it as HOCD (hypochlorite-oxidized cysteinyl-dopamine).

Hypochlorite Converts Cysteinyl-dopamine into a Redox Cycling Product

There have been reports suggesting that cysteinyl-dopamine causes oxidative stress (82,125). To investigate this, we tested cysteinyl-dopamine and HOCD for redox cycling activity. A convenient assay for determining the redox cycling activity is to
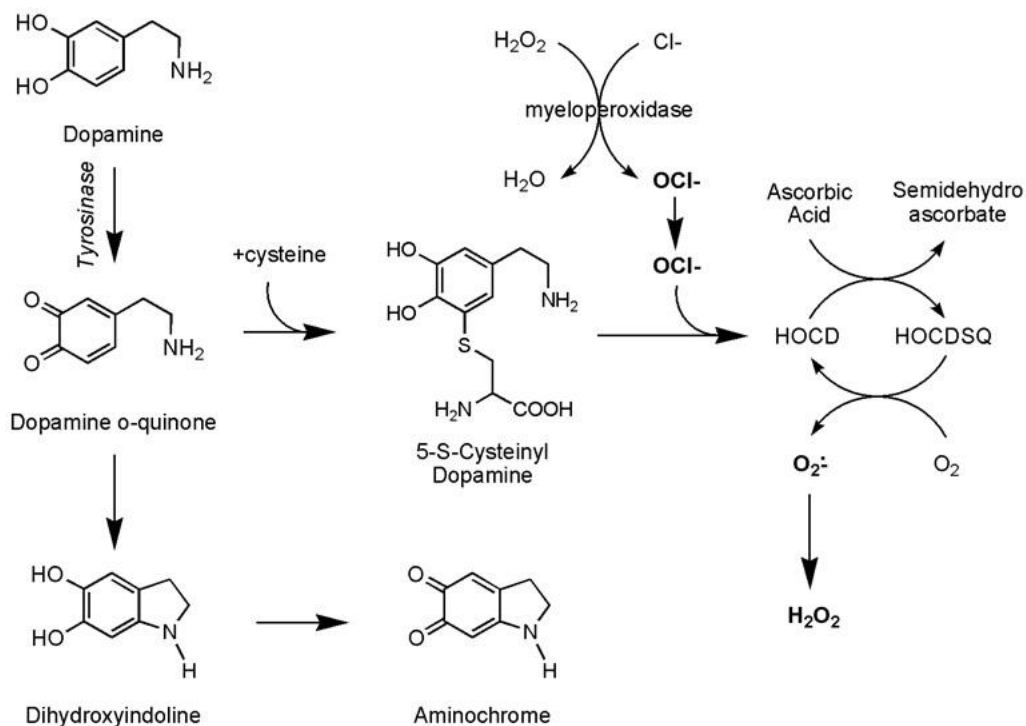
Figure 7. Non-enzymatic dopamine oxidation reactions – HOCD and HOCDSQ are the oxidized and semi-quinone forms of the redox cycling compound formed by reaction of cysteinyl-dopamine with hypochlorite.

measure the rate of ascorbate-dependent oxygen consumption using a Clark-type oxygen electrode. The compound is reduced by ascorbic acid and re-oxidized by molecular oxygen. To recapitulate the dopamine oxidation process, dopamine oxidation was accelerated using tyrosinase to form the o-quinone. This produces cysteinyl-dopamine in the presence of cysteine and aminochrome in its absence. Using the ascorbic acid-dependent oxygen consumption assay, redox cycling is not observed when 25 µM dopamine is oxidized by tyrosinase in the presence of cysteine unless hypochlorite is present (Fig. 9A). Cysteinyl-dopamine itself therefore is not effective at redox cycling, but is efficiently converted into a redox cycling product when oxidized by hypochlorite. In the presence of hypochlorite, all of the O_2 in the solution is consumed within minutes after adding an excess of ascorbic acid. Since the oxygen concentration exceeds that of

24 hours

48 hours

72 hours

24 hours

D)

48 hours

E)

72 hours

F)
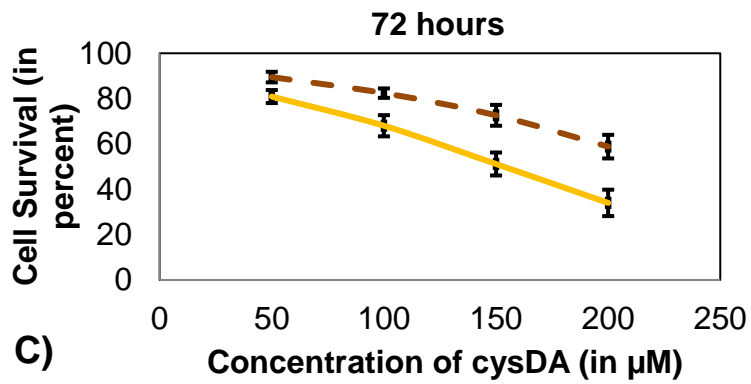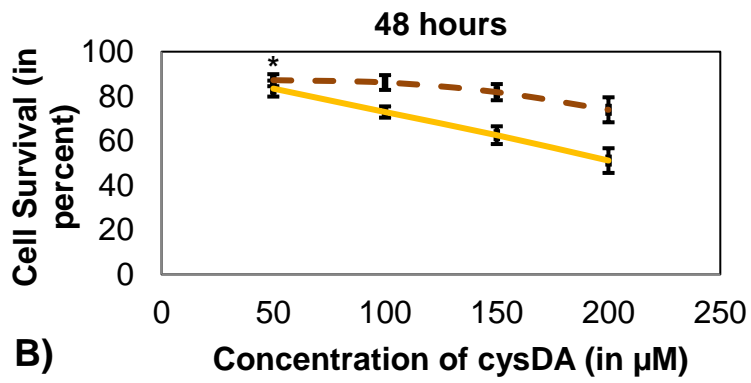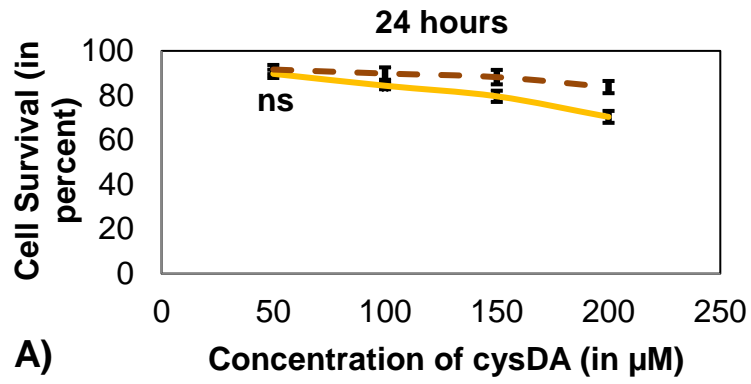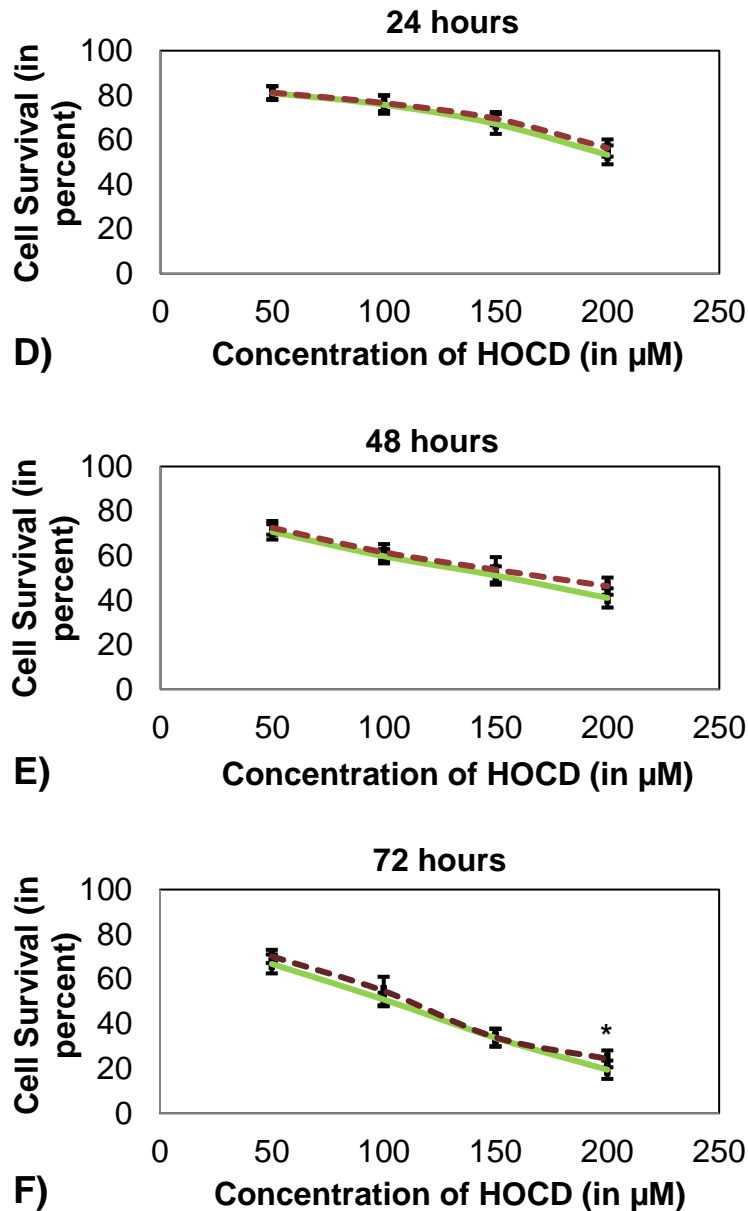
Figure 8. Taurine reduces toxicity of cysteinyl-dopamine but not of HOCD in PC12 cells – PC12 cells were treated with cysteinyl-dopamine or HOCD at the indicated concentrations and both live and dead cells were counted after 24, 48 or 72 h. Dashed lines show the effect of 25 mM taurine added to the medium. Each point is the average (± standard deviation) of nine replicate samples. For cysteinyl-dopamine (A, B and C), survival with taurine was significantly greater than survival without taurine with $p < 0.0001$ in all cases except 50 µM cysDA at 24 h (p=0.054) and 48 h (p=0.023) as indicated. For HOCD (D, E and F), survival with taurine was not significantly different from without taurine (p > 0.05) except for 200 µM HOCD at 72 h (p=0.018).
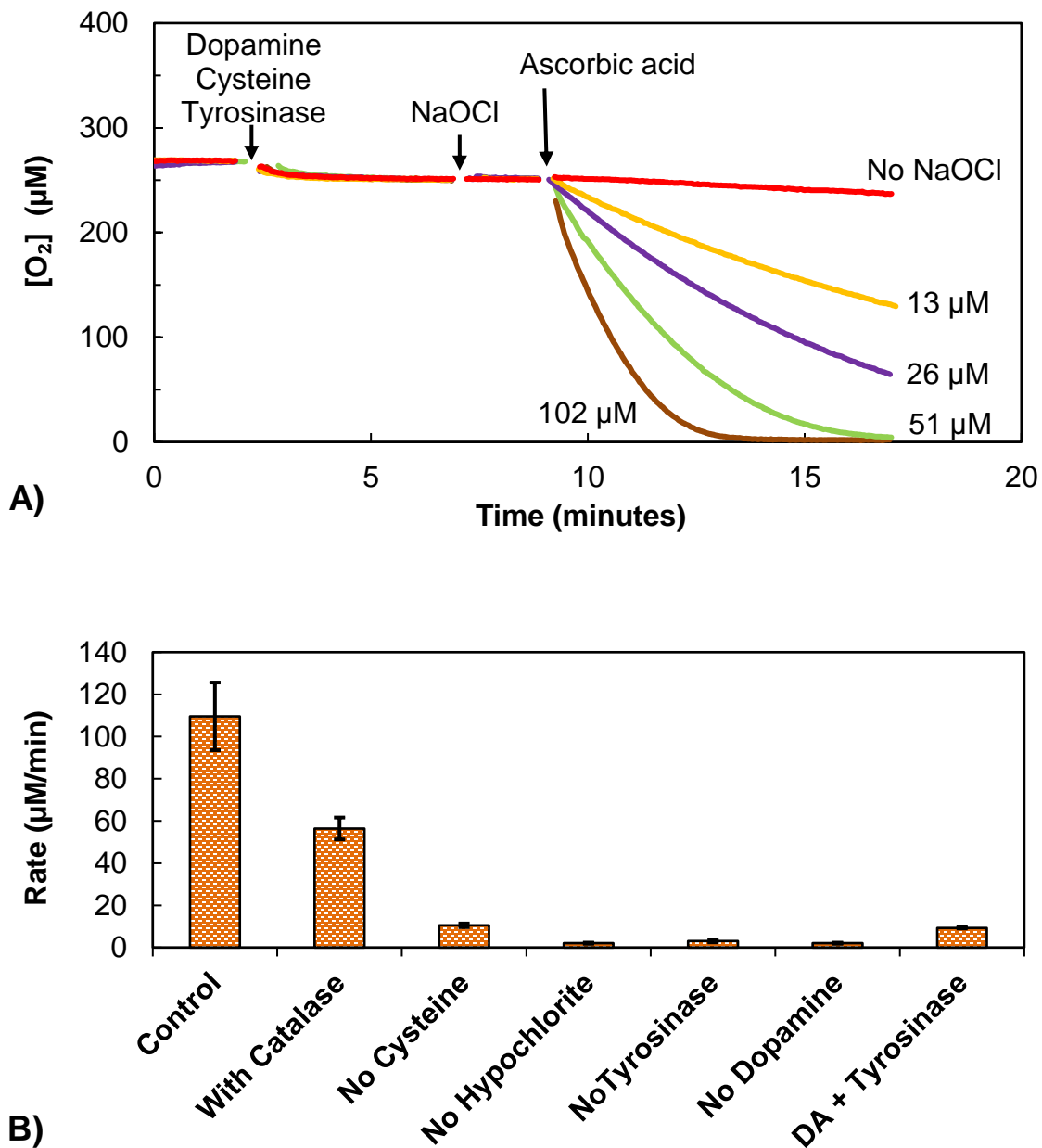
Figure 9. Conversion of dopamine into redox cycling products by hypochlorite – (A) Redox cycling was monitored as O_2 consumption at 37 °C and pH 7.4. Redox cycling products were formed by adding dopamine (25 µM), cysteine (37.5 µM) and tyrosinase (100 units) followed by the indicated concentration of sodium hypochlorite. Redox cycling was initiated by adding 2.5 mM ascorbic acid. (B) Rate of redox cycling by dopamine oxidation products. Rates were determined from the initial slope following ascorbic acid addition from recordings obtained as in A using 100 µM sodium hypochlorite. Catalase (100 µg) was added to the indicated sample at the same time as ascorbic acid. For each of the other conditions, indicated components were omitted (cysteine and sodium hypochlorite were both omitted from DA+Tyrosinase). Bars show the average ± standard deviation of three replicate samples.

dopamine by a factor of ten, the dopamine product must be cycling, alternately reduced by ascorbic acid and oxidized by O_2. Detectable redox cycling may be observed with hypochlorite concentrations as low as a few micromolar. Dopamine treated with tyrosinase in the absence of cysteine produces aminochrome which has weak redox cycling activity (Fig. 9B). This is observed both in samples with no cysteine and in those with just dopamine and tyrosinase. Dopamine like cysteinyl-dopamine has no redox cycling activity itself. Catalase, which converts H_2O_2 to ½ O_2 + H_2O, reduces the rate of O_2 consumption by approximately 50% confirming that H_2O_2 is the ultimate product of redox cycling.

Superoxide is Produced by Cells Treated with Redox Cyclers

Apart from the ability to redox cycle, we have shown that HOCD is toxic to PC12 cells at micromolar concentrations. If these two attributes are related to each other, then we would expect to see generation of reactive oxygen species in cells following HOCD treatment. Although hydrogen peroxide is the final product of redox cycling, O_2 is typically reduced first to superoxide before disproportionating to H_2O_2. We used MitoSOX Red fluorescence assay to monitor superoxide production in PC12 cells. A visible increase in fluorescence was seen within ten minutes of adding HOCD to the cells (Fig. 10). This effect was mimicked by other known redox cyclers, 3-methyl-5-anilino-o-quinone (3MAQ) and 9,10-phenanthrenequinone. Cysteinyl-dopamine also causes this fluorescence increase but only after about 30 minutes of exposure consistent with its time-dependent conversion to HOCD. Neither dopamine nor NaOCl when added separately caused this effect, nor did it occur if the dopamine product was made without either dopamine or hypochlorite.
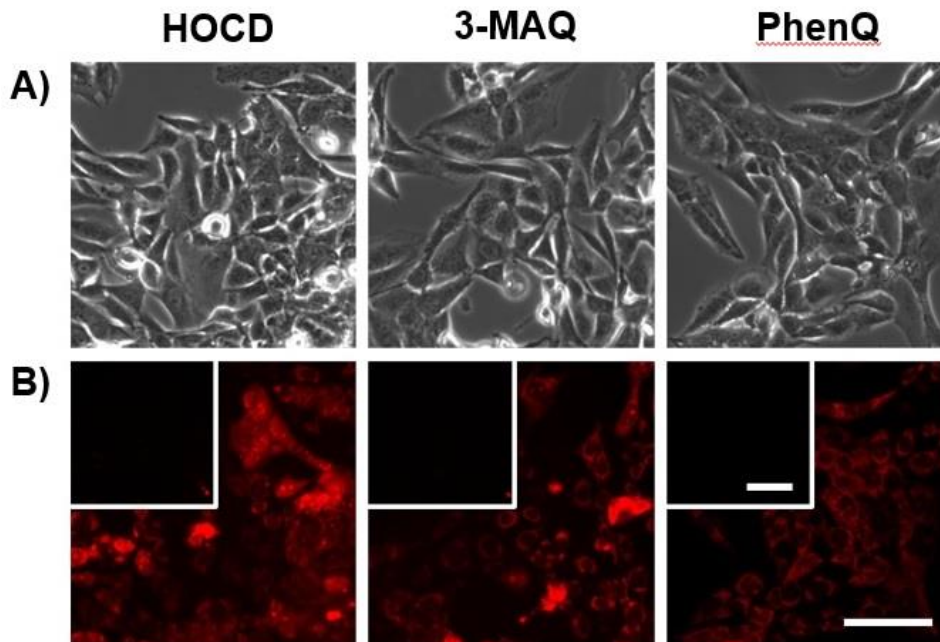
Figure 10. Superoxide generation by redox cyclers in PC12 cells – PC12 cells were grown in a 35-mm diameter imaging chamber and treated with MitoSOX Red. A bright-field image (A) was obtained followed by fluorescence images (B) before (inset) and after addition of the indicated redox cycler (125 µM HOCD, 10 µM 3MAQ, or 10 µM 9,10-phenanthrenequinone). Magnification bars = 40 µm.

Hypochlorite is Unique in its Ability to Convert Cysteinyl-dopamine into a Redox Cycler

Hypochlorite is a strong oxidizing agent, and it is possible that its effect on cysteinyl-dopamine is a consequence of simple oxidation. However, we can discard that possibility based on the failure of other common oxidants to mimic its effect, thus making hypochlorite unique in this respect (Fig. 11A). Hypochlorite at a concentration of 100 µM initiates a very rapid rate of redox cycling. Comparatively, ferricyanide produces a small amount of redox cycling activity, whereas ferric ion, perchlorate and methyl viologen (paraquat) are all ineffective. Hydrogen peroxide by itself has a negligible effect, even at
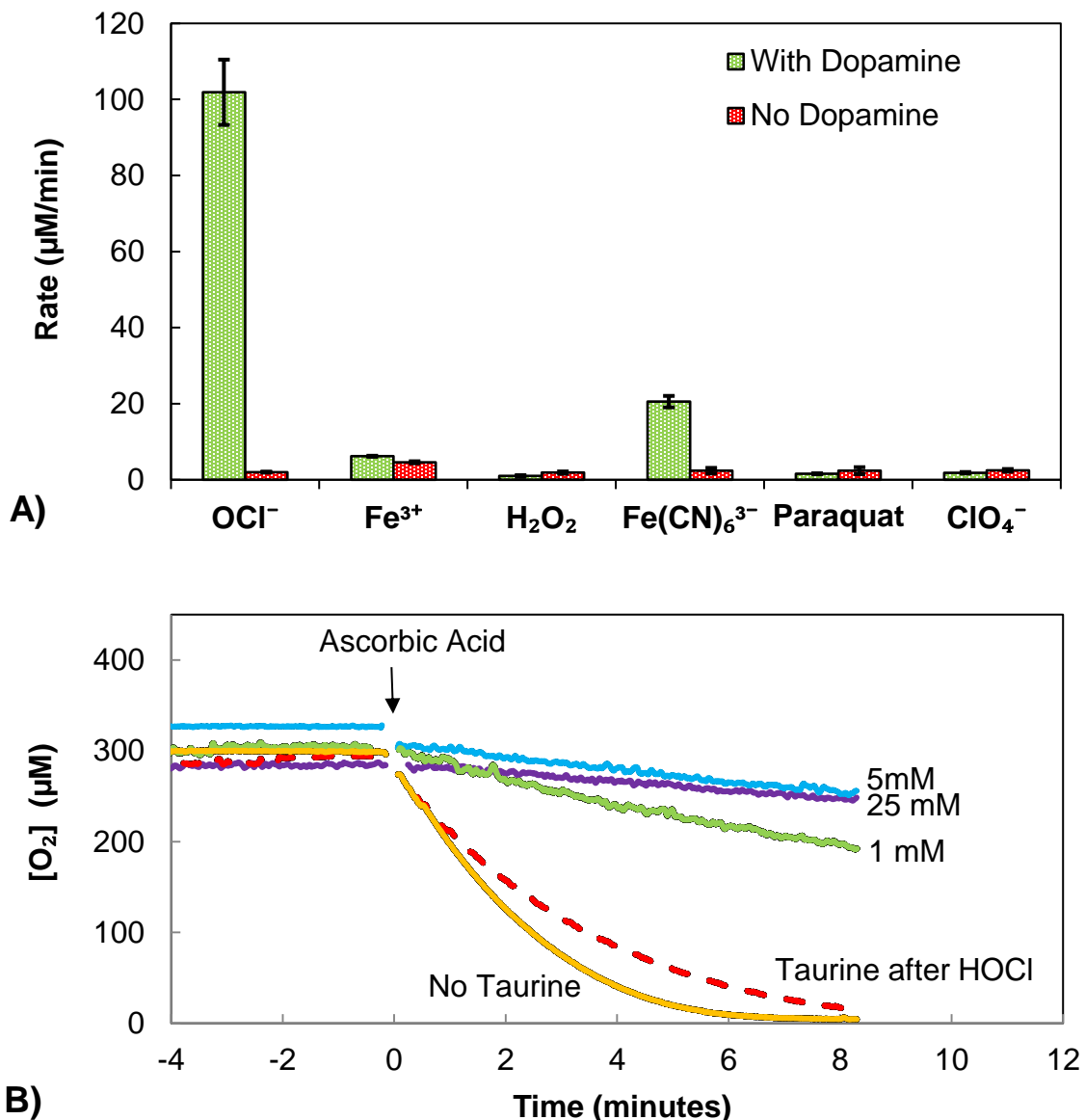
Figure 11. Sodium hypochlorite is uniquely effective at converting cysteinyl-dopamine into redox cycling products and is blocked by taurine – (A) Initial rates of O_2 consumption, recorded as in Fig. 9, were measured in the presence (green) or absence (red) of 25 µM dopamine along with cysteine and tyrosinase. Oxidants tested were sodium hypochlorite (100 µM), ferric chloride (200 µM), hydrogen peroxide (1.25 mM), potassium ferricyanide (200 µM), paraquat (200 µM) and potassium perchlorate (200 µM). Bars show the average ± standard deviation of three replicate samples. (B) Redox cycling was monitored as in Fig. 9A, but the indicated concentration of taurine was added before adding NaOCl. The dashed line shows a sample in which 25 mM taurine was added after NaOCl and just prior to ascorbic acid.

a very high concentration (>1 mM). Since taurine protects against the conversion of cysteinyl-dopamine to the toxic product HOCD in PC12 cells, we expected its effect on redox cycling to follow suit in the oxygen consumption assay. Indeed, the redox cycling activity was inhibited when taurine preceded hypochlorite addition (Fig. 11B). Addition of taurine after hypochlorite showed very little change in the redox cycling activity which not only indicates that it does not interfere with the redox cycling assay but also reaffirms that taurine has no effect on HOCD.

Myeloperoxidase Can Produce the Hypochlorite Required to Convert Dopamine into a Redox Cycler

Myeloperoxidase catalyzes the reaction of Cl^- and H_2O_2 to produce hypochlorite and water. Therefore, substituting hypochlorite with myeloperoxidase and its substrate hydrogen peroxide should replicate its effect of converting cysteinyl-dopamine into a redox cycler. Indeed, when hydrogen peroxide was provided as substrate for myeloperoxidase, ascorbate-dependent redox cycling occurred with a rate proportional to H_2O_2 concentration (Fig. 12). Furthermore, dopamine, hydrogen peroxide, myeloperoxidase and Cl^- are indispensable for producing the redox cycling product (Fig. 12B). Myeloperoxidase has both peroxidase and chlorination activities, but hypochlorite is produced by the chlorination reaction. Therefore, to check if myeloperoxidase is present in PC12 cells to convert cysteinyl-dopamine into HOCD, we tested myeloperoxidase both by Western blotting and by chlorination activity. Using both measures, a low basal level of myeloperoxidase is found in PC12 cells, and this increases proportionately when cells are exposed to varying concentrations of cysteinyl-dopamine or HOCD (Fig. 13).
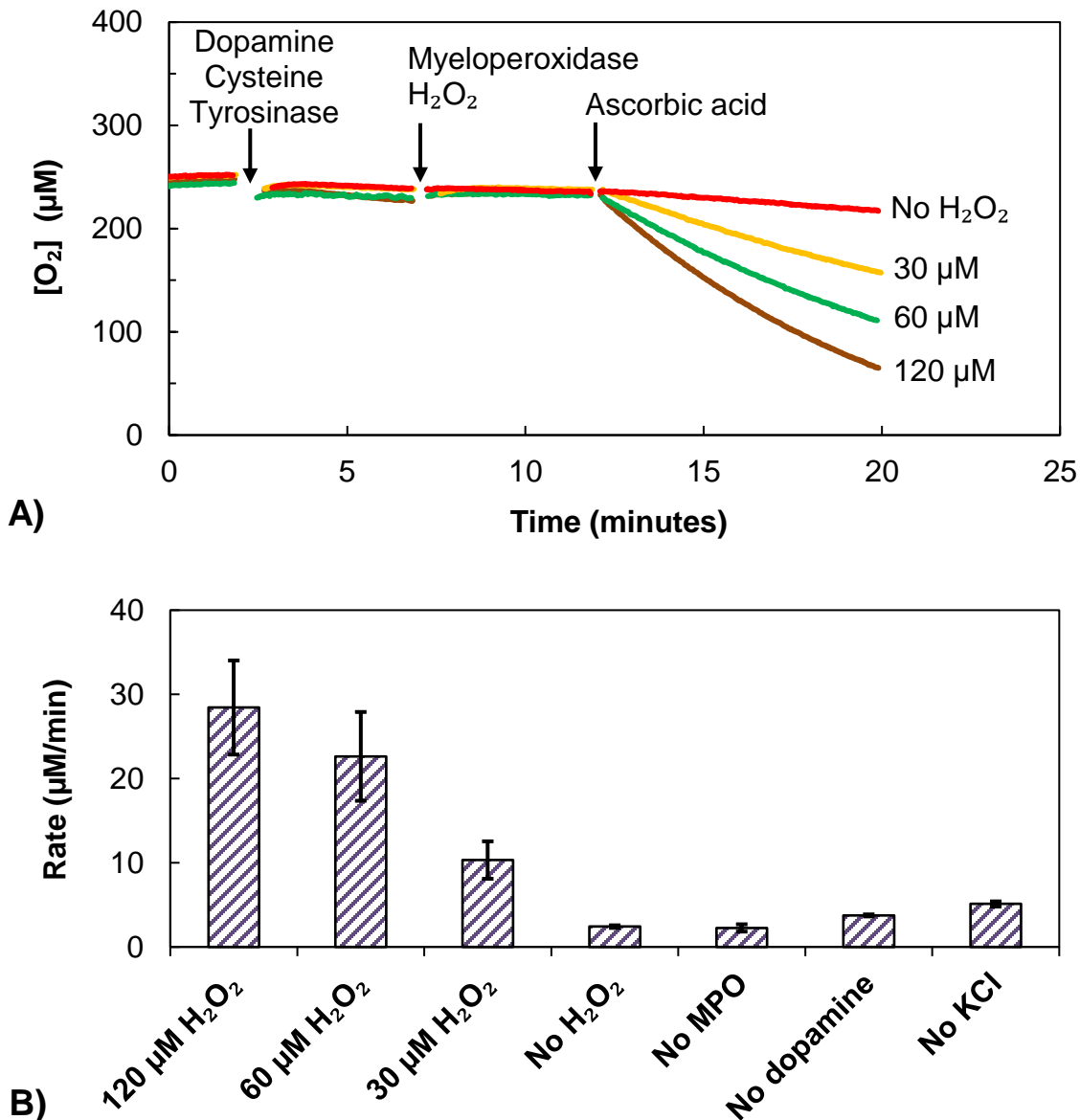
Figure 12. Conversion of dopamine into redox cycling products by myeloperoxidase and H₂O₂ – (A) Samples received dopamine (25 µM), cysteine (37.5 µM) and tyrosinase (100 units) followed by the indicated concentration of hydrogen peroxide and 6.7 µg of myeloperoxidase in 0.1 M KCl. Redox cycling was initiated by adding 2.5 mM ascorbic acid. (B) Rate of redox cycling by products of myeloperoxidase/H₂O₂ treatment. Rates were determined as the initial slope following ascorbic acid addition from recordings obtained as in (A). Samples lacking myeloperoxidase (MPO), dopamine and KCl were obtained with 120 µM H₂O₂. Bars show the average ± standard deviation of three replicate samples.
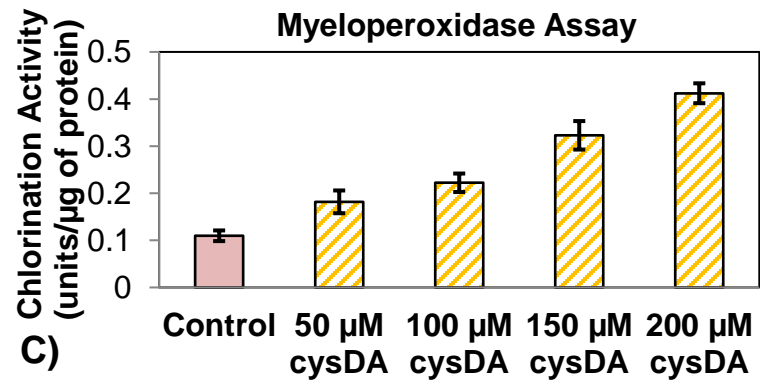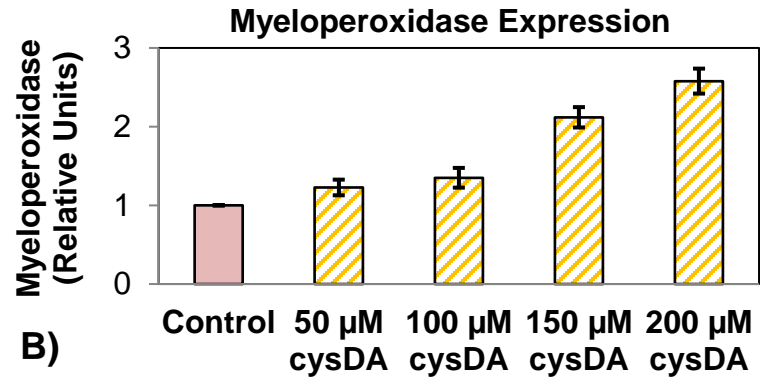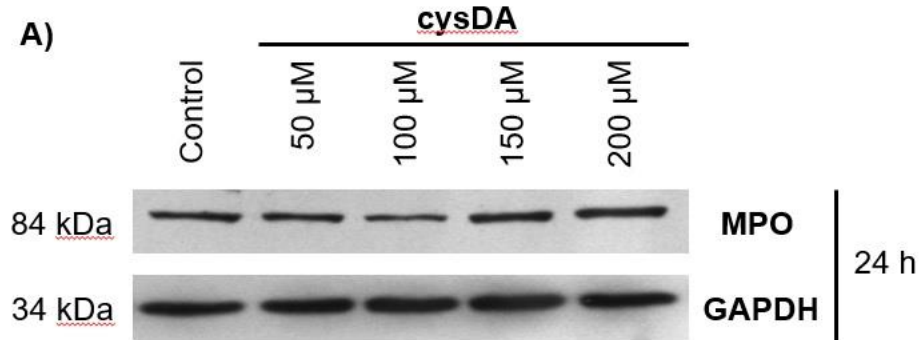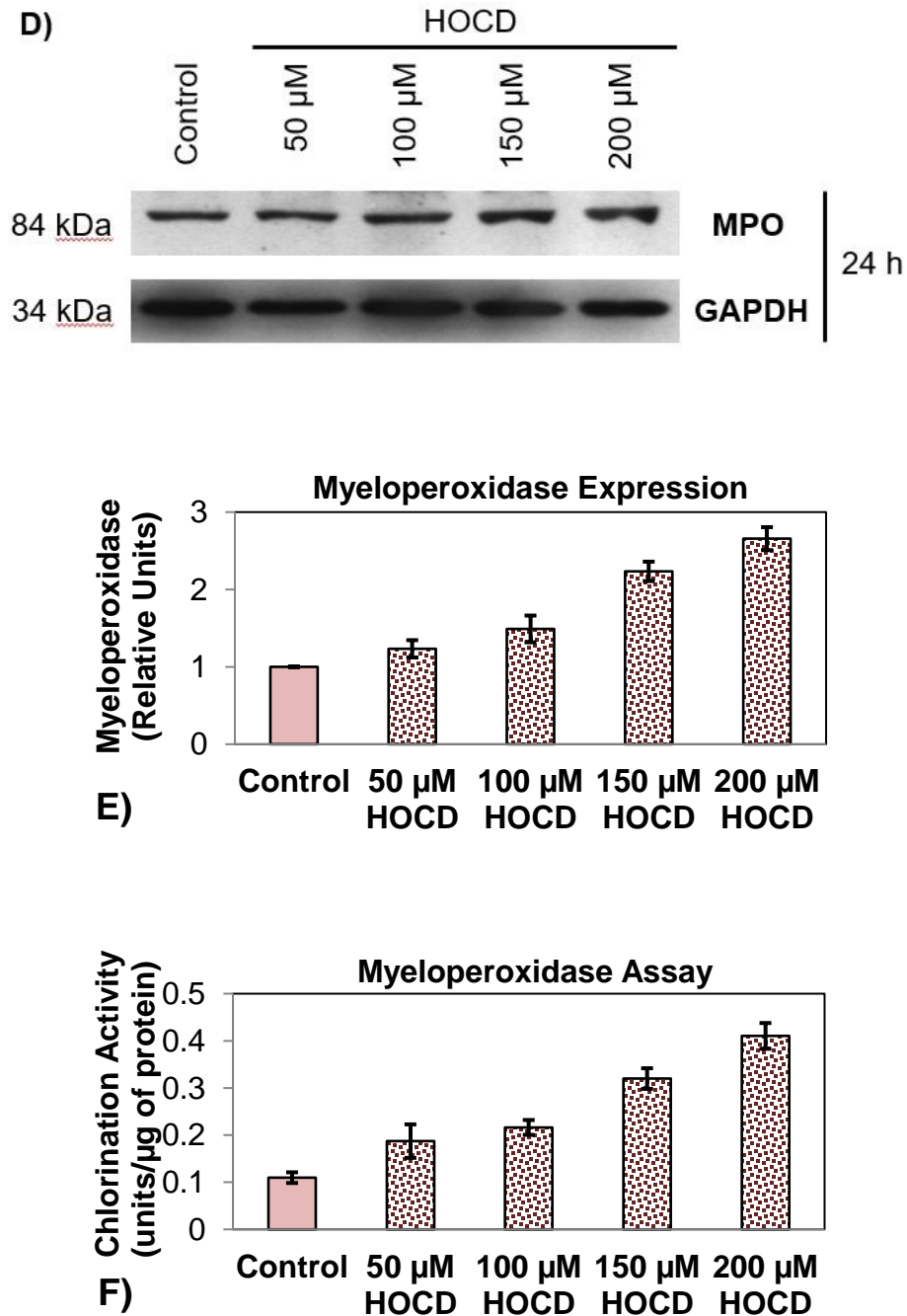
Figure 13. Cysteinyl-dopamine and HOCD increase myeloperoxidase expression and activity – PC12 cells were treated with cysteinyl-dopamine or HOCD at the indicated concentrations for 24 h. Extracts were tested for myeloperoxidase expression by Western blotting (A and D) and assayed for chlorination activity (C and F). Quantification of Western blots are shown in B and E. Bars show the average (± standard deviation) of three replicate samples.

It has also been reported that rotenone leads to increased expression of myeloperoxidase in neurons and glial cells (129-131). With low concentrations of cysteinyl-dopamine or HOCD, 10 nM rotenone causes a marked increase in the level of myeloperoxidase (Fig. 14). Concomitantly, rotenone significantly reduces survival of PC12 cells treated with cysteinyl-dopamine. On the other hand, addition of rotenone has no effect on the greater initial toxicity of HOCD. This differential effect of rotenone suggests that it is enhancing the conversion of cysteinyl-dopamine to HOCD. Therefore, the dopaminergic toxicity of rotenone is likely due, at least in part, to its effect on myeloperoxidase expression.

A)

Myeloperoxidase Expression

B)

Myeloperoxidase Assay

C)

D)

50 µM HOCD

Control | - Rotenone | + Rotenone

84 kDa   MPO

34 kDa   GAPDH

24 h

Myeloperoxidase Expression

E)

Myeloperoxidase Assay

F)
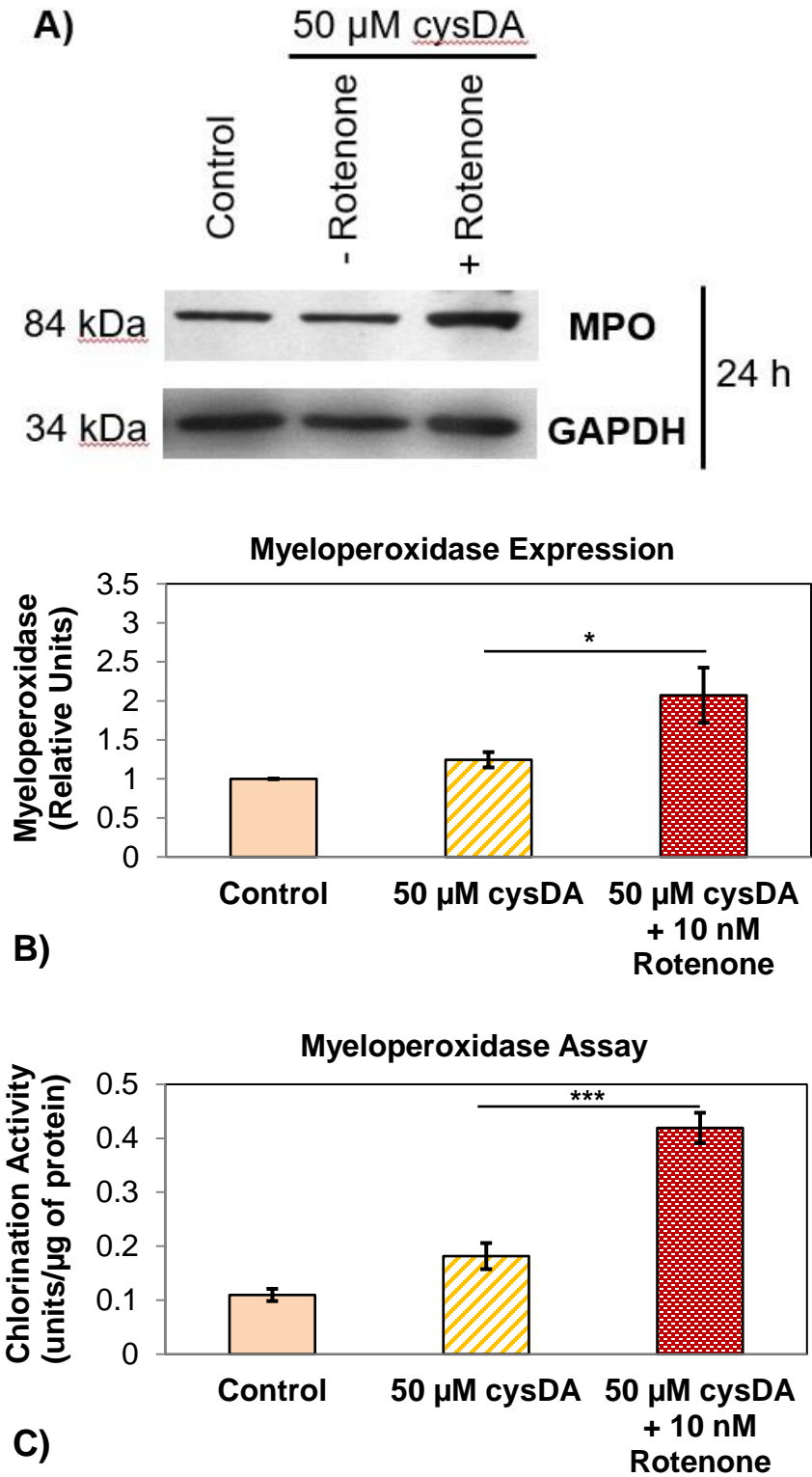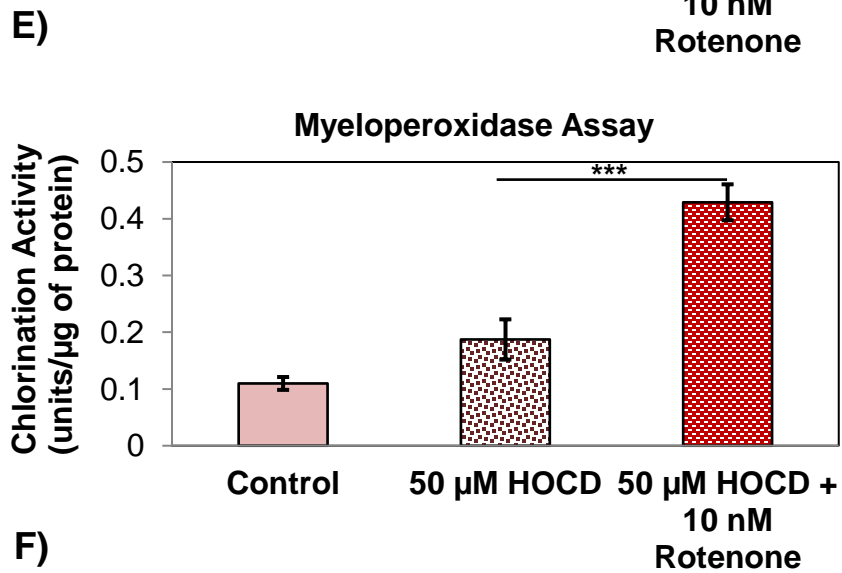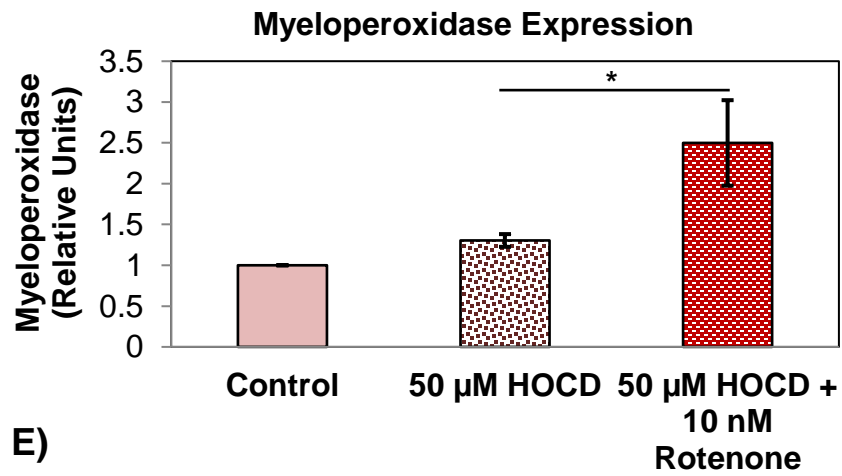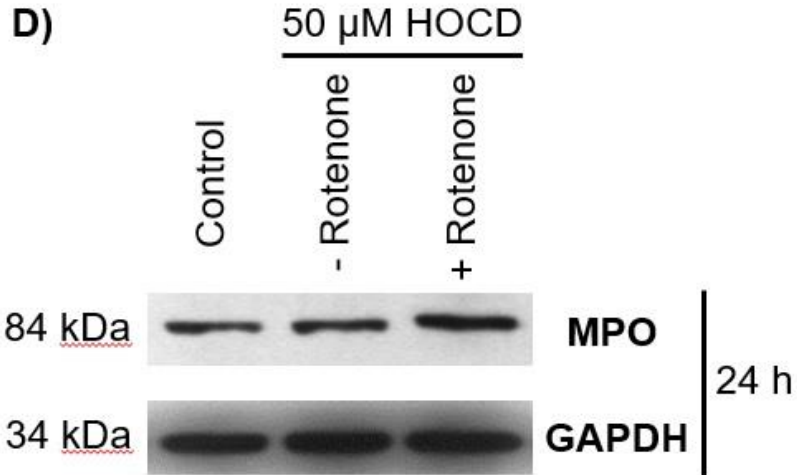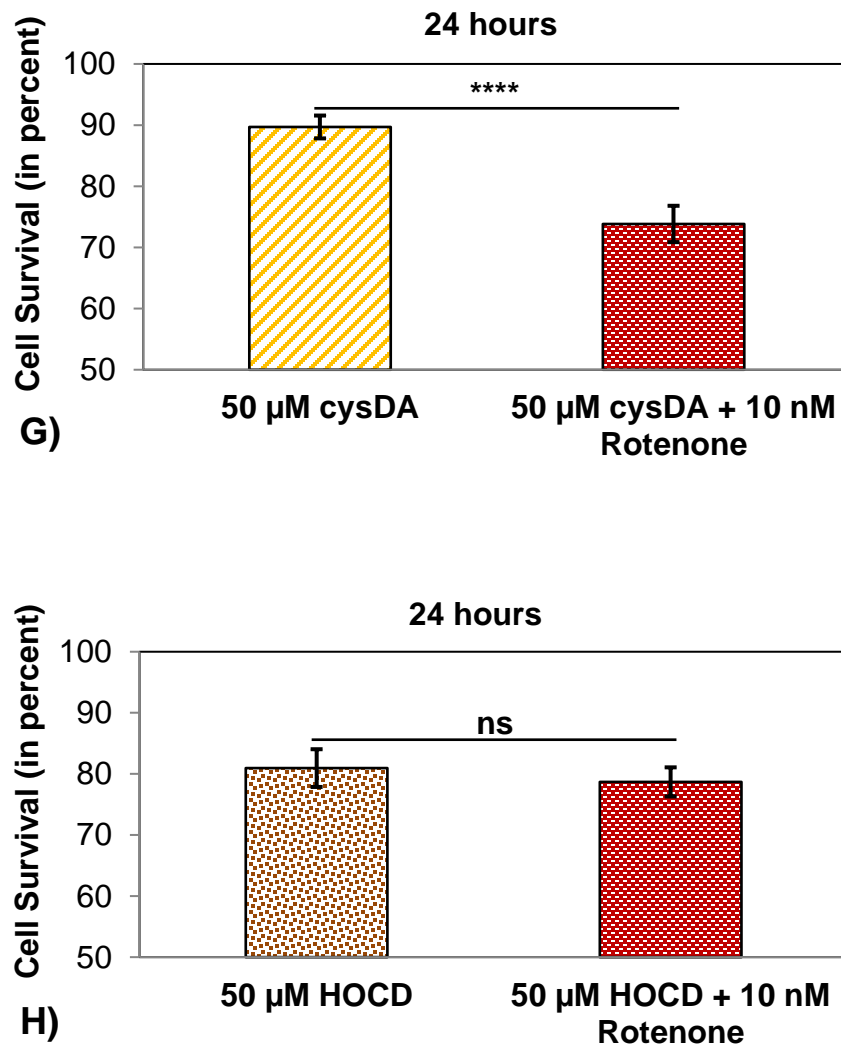
Figure 14. Rotenone increases myeloperoxidase and enhances toxicity of cysteinyl-dopamine but not of HOCD – Myeloperoxidase was assayed both by Western blotting (A and D) and by chlorination activity (C and F) after 24 h of treatment with 50 µM cysDA or 50 µM HOCD in the presence or absence of 10 nM rotenone. Quantification of Western blots are shown in B and E. Toxicity was also measured after 24 h of these treatments (G and H). Bars show the average (± standard deviation) of 3 (myeloperoxidase expression), 4 (myeloperoxidase activity), or 9 (toxicity) replicate samples.

DISCUSSION

Although the role of dopamine in Parkinson's disease has been questioned (132), there is good evidence that dopamine oxidation contributes to neurotoxicity (15,133-136). Studies have suggested that dopamine oxidation metabolites damage mitochondria and contribute in a major way to the death of dopamine neurons. For example, dopamine oxidation by tyrosinase enhances the toxicity of α-synuclein, particularly the mutant form associated with Parkinson's disease (15,135), and tyrosinase-treated dopamine also triggers the mitochondrial permeability transition in isolated brain mitochondria (14). On the contrary, increased dopamine and increased L-DOPA, instead of promoting oxidation of dopamine, do not seem to accelerate the progression of Parkinson's disease (132). Some of this uncertainty may arise because dopamine oxidation yields different products, so it is important to understand which are destructive and the physiological conditions under which their formation is favored.

Considering non-enzymatic oxidation, in the absence of thiol groups, the oxidation of dopamine to dopamine-o-quinone may be followed by the insertion of the side chain amine in to the o-quinone to form the bicyclic product aminochrome and its metabolite 5,6-dihydroxyindole (54,137,138). These dopamine oxidation products are also major components of neuromelanin, the pigment giving the substantia nigra its dark color. Some attention has been focused on the toxicity of these products (139-141), but they are highly unstable under physiological conditions. The cyclization of the dopamine quinone is not likely to compete with cysteine because it is a slow process (137). Thus dopamine oxidation is likely to form a thiol adduct, either with cysteine or glutathione. Competition between glutathione and cysteine for reaction with the dopamine quinone is likely to be

more significant, and it is noteworthy in this connection that Parkinson's patients typically have a low concentration of glutathione in the substantia nigra. Also the dopamine/glutathione adduct may also be metabolized to cysteinyl-dopamine, making it the predominant product.

Treatment of cells with cysteinyl-dopamine can result in oxidative damage, a rise in intracellular calcium, and ultimately apoptosis (82,125,126). Recently, Vauzour et al. (127) attributed its toxicity to combined effects of cysteinyl-dopamine itself and DHBT-1 (7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2H-1,4-benzothiazine-3-carboxylic acid). Dryhurst and colleagues (80,142,143) have shown that DHBT-1 is the principal product formed by the air oxidation of cysteinyl-dopamine. It inhibits mitochondrial Complex I, but it is a weak cytotoxic agent requiring millimolar concentrations to be effective. A major source of ambiguity here is that a specific mode of action has not been determined for any of these compounds.

Our results show that cysteinyl-dopamine is toxic to PC12 cells only once it is oxidized by hypochlorite; cysteinyl-dopamine by itself may not cause much damage (Fig. 8). The product formed by hypochlorite treatment has strong redox cycling activity and ability to thereby generate reactive oxygen species. A potent redox cycler may also be obtained by treating the 4-methylcatechol/cysteamine product, which lacks the amine of dopamine and the carboxyl of cysteine, with hypochlorite. This suggests that the catechol ring of dopamine and the amine and thiol of cysteine are required to form the redox cycling product. We have also found that 5-amino-o-quinones, like 3-methyl-5-anilino-o-quinone and aminochrome, are active redox cycling compounds. We suggest, therefore, that hypochlorite converts cysteinyl-dopamine into a compound incorporating this structure by

facilitating attack of the cysteinyl amine on C6 of dopamine. This internal rearrangement of cysteinyl-dopamine would make HOCD a 7,8-dihydroxy-1,4-benzothiazine. Indeed, NMR and mass spectra of the 4-methylcatechol/cysteamine product support this structure.

The concentrations of dopamine, cysteine and hypochlorite needed to generate an active redox cycler are physiologically reasonable (Fig. 9). Although concentrations used in our toxicity studies have been relatively on the higher side (Fig. 8), there are a number of reasons that need to be considered in this regard. First, formation and toxicity of HOCD may be reduced in the presence of protective compounds such as glutathione and taurine, both of which have been reported to be low in Parkinson's patients (144,145). Second, the permeability of cysteinyl-dopamine and HOCD may limit their toxicities. It is possible that intracellular generation of these compounds may increase effective toxicity compared to exogenous addition. Third, HOCD concentrations specified in this work assume a 100% yield. Since the yield is undoubtedly less than this, toxicity may occur at correspondingly lower concentrations. Finally, Parkinson's disease is a progressive movement disorder which develops over decades, so slow accumulation of damage caused by low concentrations of these compounds may be sufficient.

Like other neurodegenerative disorders, Parkinson's disease progresses slowly and symptoms are usually prominent only at later stages of life. Therefore, it is very difficult to analyze the subtle physiological changes occurring at molecular and cellular levels over the course of disease progression. To understand the etiology of Parkinson's disease several models depicting the cellular changes underlying the disease have been developed, but how well the model mimics the pathophysiology of PD has been the

persisting question. Amongst the different models for Parkinson's disease, the rotenone model is very intriguing (45). Rotenone is a non-selective inhibitor of Complex I of the mitochondrial electron transport chain and has been found to mimic mitochondrial dysfunction in PD. Mitochondrial dysfunction is known to play a role in many neurodegenerative diseases, yet remarkably rotenone only models Parkinson's disease. However, the question how rotenone specifically leads to the death of dopamine neurons, still remains to be answered.

Interestingly, recent studies have shown that very low concentrations of rotenone lead to increased myeloperoxidase expression. These reports hint that rotenone upregulates myeloperoxidase in microglia, and this contributes to the vulnerability of dopamine neurons (129,130). Also, myeloperoxidase is significantly higher in postmortem tissue from the ventral midbrain of PD patients (131). These observations suggest a possible role for myeloperoxidase (MPO) and its product hypochlorite in the rotenone model as well as in Parkinson's disease. Our results with PC12 cells indicate that neuronal myeloperoxidase may produce enough hypochlorite to convert cysteinyl-dopamine to HOCD, but it is also possible that microglial MPO plays a significant or even dominant role *in vivo*. How rotenone increases myeloperoxidase expression is still not clear. It is possible that this effect on myeloperoxidase is a downstream effect of the inhibition of complex I. Or rotenone could have an unrecognized second mode of action independent of mitochondrial complex I inhibition.

The effects of hypochlorite presented here integrate many observations and provide a new perspective for Parkinson's disease (Fig. 7). Myeloperoxidase produces hypochlorite which oxidizes the dopamine oxidation product cysteinyl-dopamine to yield

HOCD. Redox cycling of HOCD produces superoxide and hydrogen peroxide creating oxidative stress. Cysteinyl-dopamine and HOCD also increase expression of myeloperoxidase, which (together with hydrogen peroxide generated by redox cycling) promotes continuous conversion of cysteinyl-dopamine to HOCD. The expected consequence would result in increased oxidative stress eventually leading to death of dopamine neurons.

Parkinson's disease affects only a small part of the population, so it is safe to assume that there are certain inherent mechanisms that protect against or repair the damage caused by oxidative stress in dopaminergic neurons. Therefore, for Parkinson's disease to develop, it is imperative to figure out which destructive events occur and which protective mechanisms fail. Another long standing issue to be probed is how dopamine oxidized compounds interact with genetic and environmental factors to contribute to the progression of the disease. The observation that hypochlorite converts cysteinyl-dopamine into a toxic redox-cycling product is not only an unappreciated link connecting dopamine oxidation, oxidative stress and the rotenone model of Parkinson's disease but may also offer a promising new approach from a therapeutic standpoint.

CHAPTER 5 – HOCD ACTIVATES THE INTRINSIC APOPTOSIS PATHWAY

INTRODUCTION

In the year 1972, the term apoptosis was described in a classic paper by Kerr, Wyllie and Currie (146). Apoptosis then was recognized as a morphologically distinct form of cell death; however, conceptual evidence of the phenomenon had been previously known (146-148). Our understanding of the detailed mechanisms involved in apoptosis stem from an elaborate study of 'programmed cell death' during the development of the nematode *Caenorhabditis elegans* (149). In this investigation, it was shown that an adult worm comes in to being from the generation of 1090 somatic cells, of which 131 cells invariably undergo 'apoptosis.' These 131 cells, irrespective of the physiological conditions, are programmed to die during the developmental process. This is consistent between worms thereby indicating remarkable control in this system. Since then, apoptosis has been widely recognized as a characteristic mode of "programmed cell death," which involves genetically controlled depletion of cells. Apart from its mechanistic approach of maintaining homeostasis, it is also involved during immune reactions or cell damage (150).

There are certain biochemical and/or morphological features that not only characterize apoptotic events but also help distinguish them from other modes of cell death. Major histological features that define apoptosis are cell shrinkage and chromatin condensation (146). Chromatin condensation is a typical feature of apoptosis during which the electron dense nuclear material aggregates around the nuclear membrane and may further result in pyknosis. DNA fragmentation is also well-known, where DNA is cleaved into small fragments. This typical feature is often referred to as "DNA laddering."

(151). Another morphological marker that usually occurs is membrane blebbing. It involves formation of apoptotic cells due to pinching off of the cell membrane. Although the cytoplasmic contents are dense, interestingly, the integrity of organelles and membrane is intact.

Unlike apoptosis, necrosis is an energy-independent degradative process where cells undergo passive death. Necrosis affects larger populations of cells unlike apoptosis wherein usually individual or clusters of cells are affected in a controlled manner. Alternative terms such as "oncotic cell death" and "oncotic necrosis" have been proposed to describe cell death by necrosis (152,153).

Necrosis requires ATP and involves direct damage to the cell membranes. Some of the major features that typically characterize necrosis are cell swelling; formation of vacuoles and loss of plasma membrane (146,153,154). Since there is complete loss of cell membrane in necrosis, an inflammatory reaction is usually elicited due to the release of cytosolic components into the surrounding tissue. Unlike necrosis, inflammatory reactions are neither associated with the apoptotic process nor with the removal mechanism. This is primarily due to the fact that the plasma membrane of apoptotic cell bodies remains intact thereby preventing any release of the cellular constituents into the surrounding tissues (155,156).

Apoptosis is primarily divided into two main pathways – the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway.

The death receptor pathway involves transmembrane receptor-mediated signaling. It involves activation of death receptors belonging to the tumor necrosis factor receptor gene superfamily such as Fas, TNFαR, DR3, DR4 and DR5 (157-162). These

receptors have a similar cytoplasmic "death domain." Some of these death domains are named after their corresponding receptor, namely, Fas-associated death domain (FADD) or TNF-α receptor associated death domain (TRADD) (157). The death signal is characteristically initiated when homologous trimeric death receptor ligands bind to their corresponding death receptors leading to their oligomerization. Among the most well studied ligand-receptor complexes are FasL/FasR and TNF-α/TNFR1 (157-162).

The intrinsic apoptotic pathways, on the contrary, are non-receptor mediated signaling pathways that produce intracellular signal targeting molecules within the cell. They are mitochondrially initiated events (163). The trigger could initiate a signal that may regulate the pathway negatively or positively. Irrespective of the type of stimulus, mitochondrial membrane changes accompany the intrinsic pathway of apoptosis. Perturbations in the mitochondrial inner membrane not only result in an opening of the mitochondrial permeability transition (MPT) pore but also loss of membrane potential eventually releasing the mitochondrial content into the cytosol (163).

In the intrinsic pathway, the mitochondrial events are regulated by the Bcl-2 family of proteins (164). Moreover, p53, the tumor suppressor protein, also has an integral part to play as it regulates multiple proteins of the Bcl-2 family (165). However, the mechanistic details of this controlled regulation are not well elucidated. The Bcl-2 family involves both pro-apoptotic as well as anti-apoptotic proteins. Both are involved with mitochondrial membrane integrity and control the release of cytochrome c from the mitochondria by regulating the outer mitochondrial membrane permeability. Among these, anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w and BAG, whereas pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. Pro-apoptotic proteins are

mostly BH123 proteins, having three Bcl-2 homology domains. However, some pro-apoptotic proteins such as PUMA, NOXA, etc., are BH3 proteins only. And anti-apoptotic proteins mostly have all four Bcl-2 homology domains. Under normal conditions, pro-apoptotic proteins are in an inactive state where they are either sequestered in the cytosol with an anti-apoptotic protein or conjugated to some other proteins inhibiting their effect. Usually reciprocal regulation exists between these two classes of the Bcl-2 family of proteins (164).

During apoptosis, the external stimulus such as any radiation or toxin, may cause either a double-stranded or single-stranded break in DNA. For example, in the case of a double-stranded break, the damage is sensed by two classes of proteins such as ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Telangiectasia and Rad3 related protein) (166,167). Both proteins are serine/threonine kinases. These proteins could further phosphorylate other serine/threonine kinases, checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2), eventually activating them (166,167). Once activated, these kinases tend to activate tumor suppressor protein p53. p53 protein under normal conditions is in an inactive state. Mdm2 is usually conjugated to p53 and marks p53 for ubiquitination. In apoptosis, once Chk1 and/or Chk2 are activated, phosphorylation of p53 prevents its conjugation to Mdm2 and therefore, p53 survives subsequent ubiquitination and proteasome degradation, and instead phosphorylated p53 forms a tetramer and initiates transcription. p53 leads to expression of many proteins such as proteins involved in DNA repair, another tumor suppressor protein p21 that arrests the cell cycle progression, and also some pro-apoptotic proteins such as Puma and Noxa (168,169).

The pro-apoptotic proteins such as Puma and Noxa, belonging to the Bcl-2 family, are found to play an important role in p53-mediated apoptosis (168). Once expressed, they tend to sequester the anti-apoptotic proteins in the cytoplasm, thereby increasing the expression of other pro-apoptotic proteins. Studies have reported an increase in the levels of BAX due to overexpression of Puma (168). This resulted in a conformational change in BAX and its translocation to outer mitochondrial membrane. Another study has shown that expression of Noxa leads to its localization to the mitochondrial membrane, resulting in inactivation of anti-apoptotic proteins (169). This will lead to the formation of the apoptosis-induced channel in the outer mitochondrial membrane and release of cytochrome c in to the cytoplasm. Once in the cytoplasm, cytochrome c encounters cytoplasmic protein Apaf-1 (Apoptotic Protease Activation factor-1) (170,171). Apaf-1 has a CARD domain (caspase recruitment domain). When cytochrome c binds to this Apaf-1 protein, it aggregates to form an apoptosome (170,171). Formation of the apoptosome recruits pro-caspases having a similar CARD domain, for example, pro-caspase-9, and auto-activates them. Once activated, these caspases further activate other executioner caspases such as caspases 3, 6 or 7. Proteins such as Smac/DIABLO and HtrA2/Omi also participate by inhibiting IAP (inhibitors of apoptosis proteins) activity (172-175).

The execution phase is the last step in the apoptosis process. Activation of caspases mark the beginning of this phase. Caspases are a group of cysteine-dependent aspartate-directed proteases. As the name suggests, caspases are proteolytic enzymes that cleave proteins at aspartic acid residues. The substrate specificities of caspases may differ among the group of caspases. Caspases usually exist in inactive forms called procaspases. All procaspases have a pro-domain, a large subunit and a small subunit.

Once activated by a signaling cascade, they are cleaved to their active intermediate form. Apart from their ability to activate other procaspases they can also aggregate and autoactivate themselves. This activation of caspases creates a protease cascade thereby amplifying the apoptotic signal, and eventually leading to cell death. Among the ones that are identified, caspases are broadly classified into two categories, initiator caspases (caspases 2, 8, 9, 10) and effector or executioner caspases (caspases 3, 6, 7). Additionally, a class of inflammatory caspases (caspase – 1, 4, 5) (176,177) and several other caspases (11, 12, 13, 14) (178-181) are also known. Of all the caspases, caspase-3 is especially significant. It can be activated by three important initiator caspases: 8, 9 and 10. Caspases-3, 6 and 7 are the effector caspases that cause the morphological and biochemical changes in the cell such as activation of cytoplasmic endonucleases that degrade nuclear material, activation of proteases that degrade nuclear as well as cytoskeletal proteins and cleavage of substrates like poly-ADP ribose polymerase (PARP), cytokeratins, the plasma membrane cytoskeletal protein alpha fodrin, etc. (182).

Our study has already shown that HOCD is toxic to PC12 cells at micromolar concentrations. However, the mechanism of cell death is not known. Therefore, the focus of this investigation was to elucidate the underlying mechanism of cell death when PC12 cells are treated with HOCD. This is important both to determine the mechanism of HOCD toxicity and to see how this correlates with the mechanism of dopaminergic neuron death in Parkinson's disease and its various chemically-induced models.

RESULTS

We have shown that HOCD and other redox cyclers lead to superoxide generation and death of PC12 cells. To elucidate the mechanism of cell death, it is important to investigate certain morphological and biochemical markers that would help differentiate between different modes of cell death. Among the morphological markers, membrane blebbing was an early candidate. During microscopic examination of PC12 cells after HOCD treatment, a remarkable change in the morphology of the cells was observed. Membrane blebs were found on the surface of a few of the cells in addition to small individual apoptotic bodies in the medium (Fig. 15). This phenomenon of membrane blebbing is a characteristic feature of apoptotic cell death.
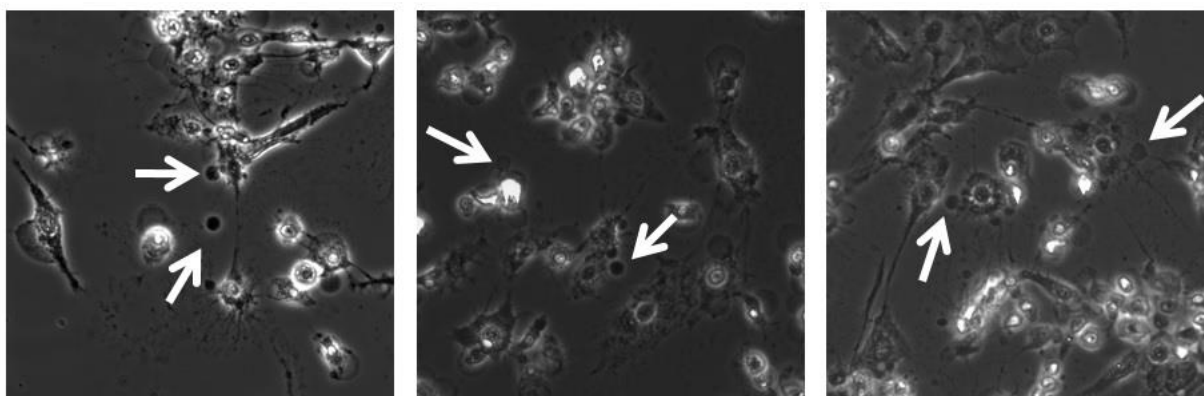
Figure 15. Formation of membrane and apoptotic blebs – Bright field microscopic images of PC12 cells taken after 24 h of treatment with 200 μM HOCD, shows the formation of membrane and/or apoptotic blebs (indicated by arrows).

To confirm that cells are undergoing death via apoptosis, we checked for the presence of cleaved poly-ADP ribose polymerase, an apoptotic marker that usually appears late in the pathway when executioner caspases are activated. By western blotting, we found increased levels of cleaved-PARP protein when PC12 cells were

treated with HOCD thereby confirming apoptosis as the selected mode of cell death (Fig. 16).

Following the observation of cleaved PARP protein, we wanted to investigate the possible involvement of mitochondria in this apoptotic process. Caspase-9 is an initiator caspase that is activated primarily after cytochrome c is released from the mitochondria. Therefore, we checked for the level of active caspase-9. We observed an increase in the amount of active caspase-9 (Fig. 17), confirming our hypothesis that the mode of cell death in PC12 cells treated with HOCD was mitochondrially driven.

Since p53 has been reported to play an important role in the mitochondrially mediated apoptotic pathway, we checked for the level of p53 in whole cell lysates of PC12 cells treated with HOCD by western blotting. Indeed, we found increased p53, further indicating involvement of mitochondria in this apoptotic process (Fig.18).
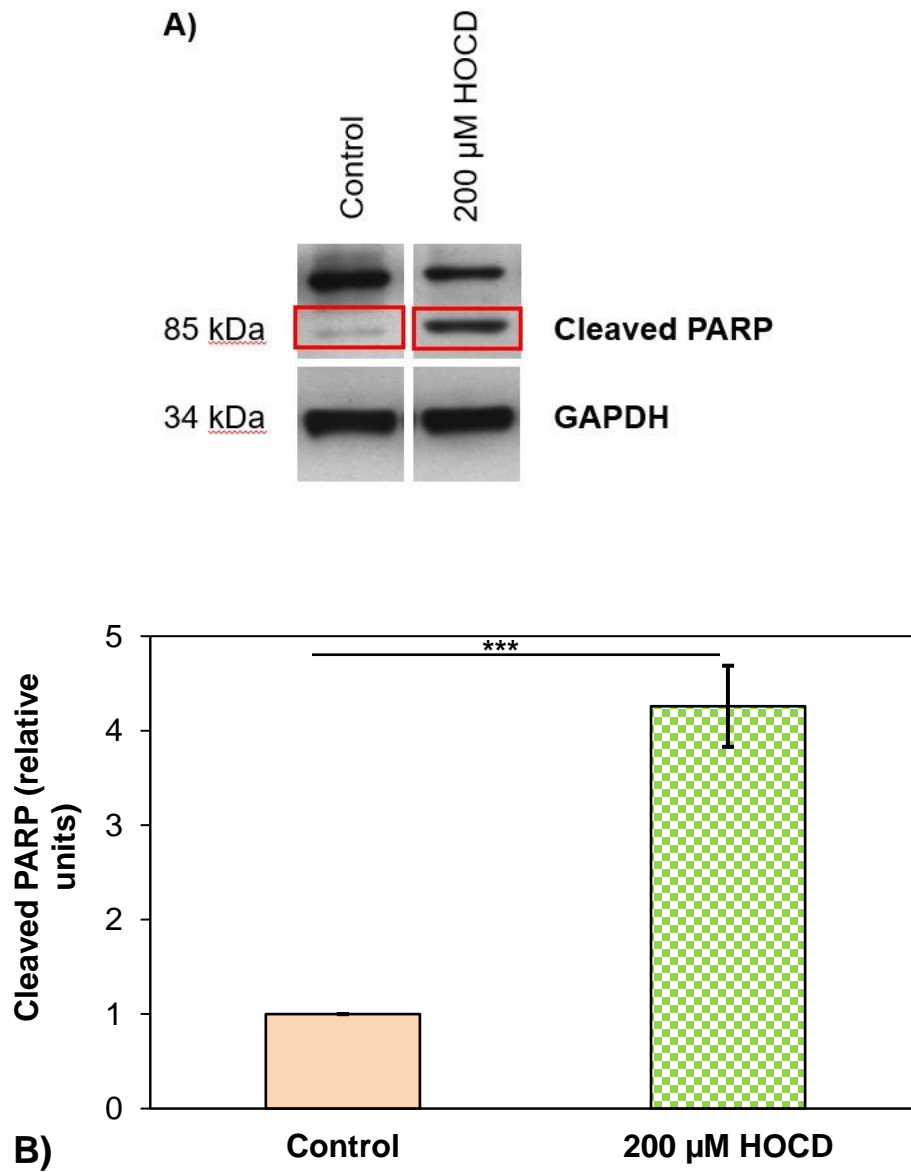
Figure 16. Increased level of cleaved poly-ADP ribose polymerase – Western blot analysis of HOCD-treated PC12 cell lysates was performed. HOCD concentration was 200 µM, and cell lysates were prepared after 72 h of HOCD treatment. Whole cell lysates were resolved using SDS PAGE and probed with anti-PARP antibody. (A) Protein levels obtained by blot analysis (A) were also quantified (B) and normalized to the loading control, GAPDH.
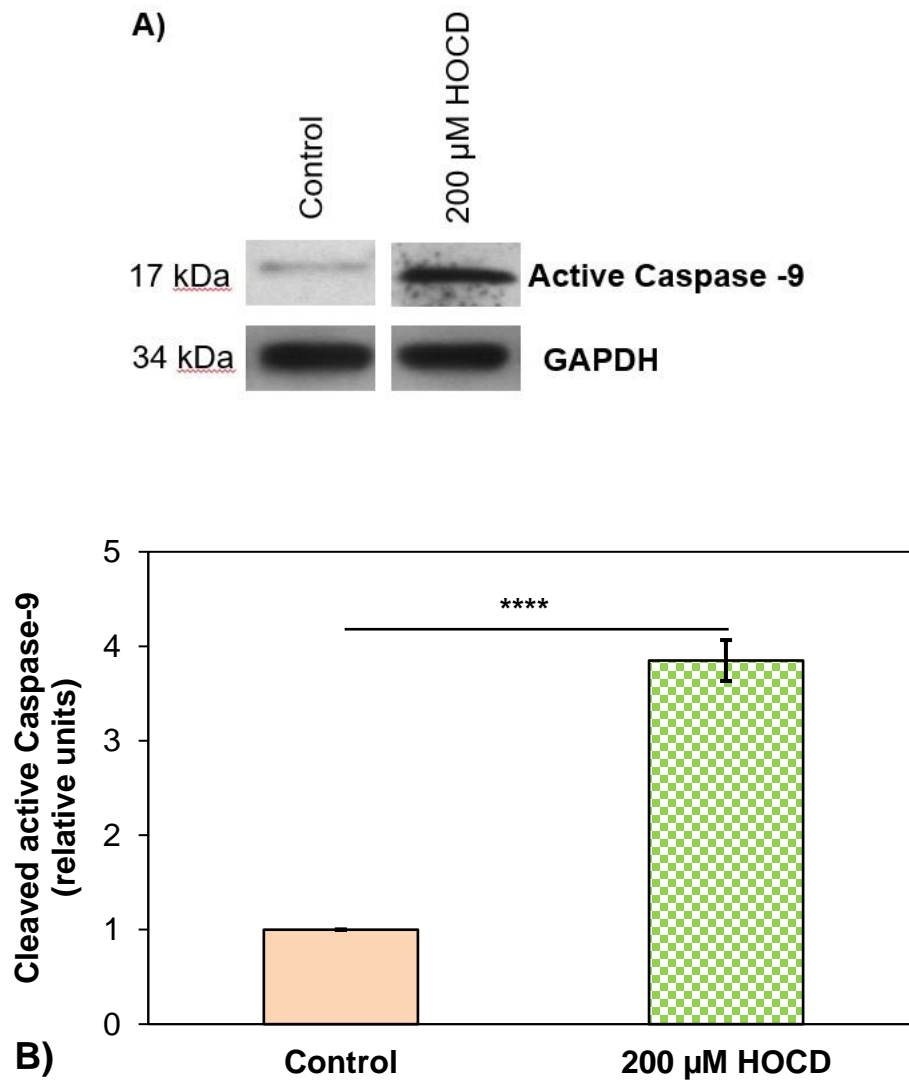
Figure 17. Increased level of active caspase-9 – Western blot analysis of HOCD-treated PC12 cell lysates was performed. HOCD concentration was 200 µM, and cell lysates were prepared after 72 h of HOCD treatment. Whole cell lysates were resolved using SDS PAGE and probed with anti-active caspase-9 antibody. (A) Protein levels obtained by blot analysis (A) were also quantified (B) and normalized to the loading control, GAPDH.
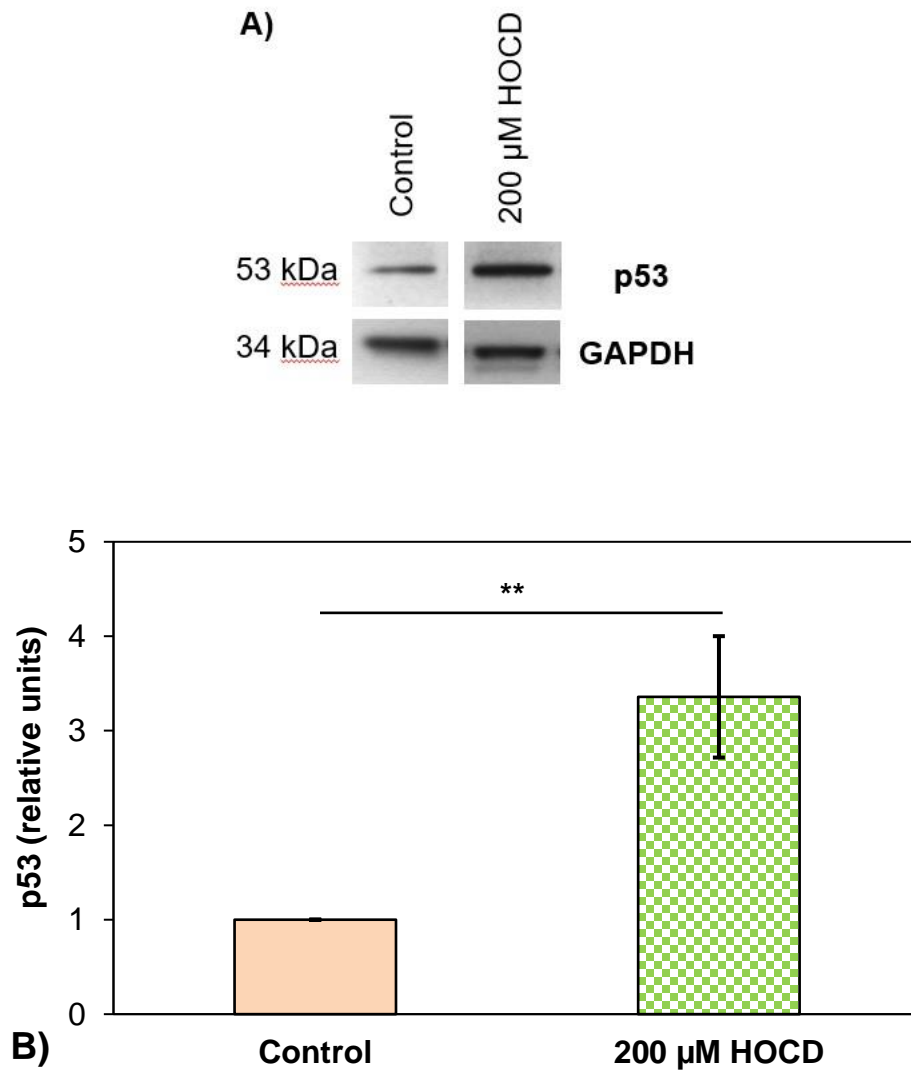
Figure 18. Level of p53, a regulator of mitochondrially-mediated apoptosis –
Western blot analysis of HOCD-treated PC12 cell lysates was performed. HOCD
concentration was 200 µM, and cell lysates were prepared after 72 h of HOCD treatment.
Whole cell lysates were resolved using SDS PAGE and probed with anti-p53 antibody.
(A) Protein levels obtained by blot analysis (A) were also quantified (B) and normalized
to the loading control, GAPDH.

DISCUSSION

In Parkinson's disease models, activation of the mitochondria-dependent apoptotic pathway has been found to be prevalent in the death of dopaminergic neurons (183,184). For instance, mitochondrial release of cytochrome c, followed by caspase-9 activation and eventual apoptotic nigral cell death was observed for the MPTP model in mice (185). These events were found to be regulated by the pro-apoptotic protein Bax (185-187). Supporting this finding, another study showed that dopamine neurodegeneration was attenuated by overexpressing the anti-apoptotic protein Bcl-2 (188,189) or by targeting caspase-9 or Apaf-1 (190,191).

Furthermore, many familial forms of Parkinson's disease have shown either direct or indirect correlation between gene mutations and the mitochondrial apoptotic pathway (192). Overexpression or aggregation of α-synuclein have been shown to induce caspase-9 and caspase-3 activation damaging dopaminergic neurons (193). Similarly, mutation in LRRK2 (194), PINK1 (195,196) and PARKIN (197) all lead to cytochrome c release.

Apoptosis has also been widely studied in mammalian cell lines and primary neuronal cultures, in regards to dopaminergic neurons and Parkinson's disease. Tabakman et al. showed increased activities of JNK and p38 in PC12 cells due to neurotrophic factor deprivation (198). Mitochondrial cytochrome c release and increased levels of activated caspase-9 were observed in PC12 cells (199). Also, in mesencephalic and MN9D primary dopaminergic neurons, cytochrome c release from mitochondria and activation of caspases were observed after 6-OHDA and MPP+ treatment (200). Similar events were recorded in MPP+ treated (201) and 6-OHDA treated SH-SY5Y cells (202).

Even in the rotenone model for PD, caspase activation was observed in human neuroblastoma cells (203), in addition to cleavage of PARP, DNA fragmentation and cell death (204).

Dopamine quinone can lead to opening of the mitochondrial transition pore due to the impairment of oxidative phosphorylation and mitochondrial swelling. This may further lead to the release of both pro-apoptotic and anti-apoptotic factors (14) as well as non-specific release of cytochrome c initiating apoptotic events (205). Due to the slow rate of neuronal death in PD, detection and assessment of mode of cell death is very difficult. Using various mechanisms, apoptotic dopaminergic neurons have been reported in substantia nigra of PD patients (206-210), where increased expression of CD95/Fas and p53 have also been detected (211).

Involvement of the extrinsic pathway in the death of dopaminergic neurons, though not predominant, has been well investigated. Expression of FasL and Fas receptor is found in neurons, astrocytes and glial cells, however, astrocytes have interestingly shown resistance to Fas-mediated apoptosis (212). Significantly higher levels of soluble Fas were first reported by Mogi et al. in nigrostriatal dopaminergic regions of PD patients (213). Hayley et al. also reported the possible connection of c-Jun-Fas signaling to the loss of dopaminergic function in the MPTP-induced PD model (214). Apart from Fas signaling, altered levels of TNF-α were detected in glial cells in substantia nigra, in cerebrospinal fluid (CSF) and brain of PD patients and also in lymphocytes in PD patients. Compared to the death receptor pathway, involvement of the mitochondrial mediated apoptotic pathway in death of dopaminergic neurons has been well documented. Although both extrinsic and intrinsic pathways of apoptosis seem to be involved in

Parkinson's disease, the intrinsic pathway is more prevalent, probably due to the energy deprivation.

Neurons undergo apoptosis because of the informative signal received from either external stimuli or internal stimuli. Intracellular signaling depends on cell type, state of differentiation and maturity as well as developmental history, whereas extracellular factors that affect cell fate are appearance and disappearance of hormones, growth factors, cytokines and cell matrix interactions. Multiple factors are involved in the execution of apoptotic cell death in PD and once these factors are activated, they all lead to ATP depletion.

However, recent studies have shown evidence of possible interplay between the two pathways, where the components involved in one pathway could influence the other. One of the examples of such cross-talk is a study showing that initiation of the apoptotic pathway via Fas receptor activation has led to mitochondrial damage via interaction between caspase-8 and the pro-apoptotic protein Bid. Also, since recent findings have shown that apoptosis is a reversible process, inhibition of caspase activity could be an important strategy from a cell survival point of view. In fact studies have shown that 6-OHDA-induced death of dopaminergic neurons was prevented in the presence of caspase inhibitor (215,216).

Our results however, do not disregard the possibility of an interplay between the extrinsic and intrinsic pathways. It is possible that caspase-8 activation could trigger mitochondrial membrane change and subsequent caspase-9 activation. However, use of mammalian cell lines does introduce some limitations. Mammalian cell lines may not offer

the physiological conditions to study the extrinsic pathway and therefore further restrict observation of its influence on or reaction to activation of the intrinsic pathway.

Another question is how the genetic factors linked to Parkinson's disease are connected to the induction of apoptosis. For some, a direct relationship is plausible. Parkin and PINK1 are involved in mitophagy, the process by which defective mitochondria are removed from the cells. Mitochondria that fail to maintain a membrane potential across the inner membrane are culled by mitophagy in a process mediated by Parkin and PINK1 (217). Therefore, it seems reasonable that a mutation in either protein would interfere with the removal of dysfunctional mitochondria and would contribute to the release of cytochrome c in to the cytoplasm and triggering of the intrinsic pathway of apoptosis.

The involvement of α-synuclein in apoptosis is more elusive. DiMaio et al. have reported that α-synuclein binds to TOM20, a mitochondrial import protein and inhibits protein import into mitochondria (218). Impairment of this system would likely lead to mitochondrial degradation and could contribute to initiation of the intrinsic pathway of apoptosis. At the same time, Parkinson's disease is characterized by α-synuclein aggregates found in Lewy bodies. How oxidative stress and mitochondrial dusfunction contribute to α-synuclein proteinopathy is not clear. It has been suggested that clearance of protein aggregates occur by autophagy or proteasomal action and that these processes compete with mitophagy for cellular components such as Bcl-2 or ubiquitin. Thus, the occurrence of α-synuclein aggregates could interfere with the clearance of defective mitochondria and predispose the cell to death by the mitochondrial pathway of apoptosis.

The picture emerging, therefore, is that HOCD causes oxidative stress which leads to mitochondrial damage and activation of the intrinsic pathway of apoptosis. Clearly, however, we are just beginning to connect the dots and understand the roles and relationship of all of the factors contributing to the death of dopamine neurons in Parkinson's disease.

CHAPTER 6 – FUTURE DIRECTIONS

Our study has shown that cysteinyl-dopamine by itself is not toxic. Rather it is the oxidized product of cysteinyl-dopamine that is toxic. We showed that hypochlorite produced by myeloperoxidase oxidizes cysteinyl-dopamine into a cytotoxic redox cycling compound, called hypochlorite-oxidized cysteinyl-dopamine (HOCD), capable of generating free radicals and thereby creating oxidative stress. Also, HOCD was found to be toxic to catecholaminergic PC12 cells at micromolar concentrations. However, since the cytotoxic effect of HOCD was tested in mammalian cell lines, it will be interesting to check its effect in primary cultured neurons and animal models.

If HOCD is found to be toxic to primary cultured neurons and/or in animal models, it will be interesting to see if HOCD treatment leads to the aggregation and accumulation of α-synuclein and ultimately formation of Lewy bodies. Similarly, following the observation of Lewy body formation, it will be important to see the effect of HOCD treatment on dopaminergic neurons. There have been several animal models developed over the years to study the etiology of Parkinson's disease, including pathologies caused by rotenone, 6-OHDA, MPTP and LPS. In addition to the α-synuclein pathology and death of dopaminergic neurons, if HOCD recapitulates the delayed, progressive symptoms of Parkinson's disease then it could be an alternative PD model. Moreover, if HOCD is the natural trigger for PD, then it will be an important tool for elucidating the events involved in the onset and progression of the disease.

Since microglial inflammation is known to play a significant role in the pathogenesis of Parkinson's disease, it is important to see what effect HOCD would have on the activation of microglia. Microglial activation is usually measured by an increase in NADPH

oxidase (NOX-2) activity. If HOCD leads to overexpression of NOX-2, in addition to the α-synuclein pathology and death of dopamine neurons, then it would be imperative to sketch out the symptomatic events in a chronological order to elucidate the underlying molecular mechanism and its timing. Furthermore, our study showed that hypochlorite, responsible for converting cysteinyl-dopamine in to a toxic redox cycler, was predominantly produced by myeloperoxidase. However, the effect of cysteinyl-dopamine in the absence of myeloperoxidase remains to be seen. Therefore, it will be important to check the effect of cysteinyl-dopamine and HOCD in primary neurons and/or animal models with inhibited myeloperoxidase activity.

The toxicity of HOCD was attributed to its ability to redox cycle and thereby generate free radicals causing oxidative stress. However, as mentioned earlier compounds capable of redox cycling *in vitro* may not necessarily redox cycle *in vivo*. Therefore, a next step would be to observe and measure the redox cycling activity of HOCD *in vivo*. Likewise, we also do not disregard the possibility that HOCD may have an additional mode of action besides redox cycling. Thus, it will be necessary to determine the protein interactions of HOCD *in vivo*. However, this may be feasible experimentally only once we deduce the structure of HOCD.

Additionally, our study has shown that taurine was able to protect PC12 cells against cysteinyl-dopamine but not HOCD. Taurine is a sulfur-containing amino acid. Since it contains a sulfonic acid group instead of a carboxyl group, it is not a typical amino acid. Although taurine was considered as a non-essential amino acid, recent studies have indicated that taurine is essential for humans. However, humans have limited capacity to synthesize taurine themselves, compared to other mammals, due to relatively low activity

of the enzyme required for a crucial transformation step in the synthesis of taurine. Taurine does have the ability to cross the blood brain barrier. Moreover, taurine is a hypochlorite scavenger which enables it to protect cysteinyl-dopamine. Since PD patients have low levels of taurine, we suspect taurine to be an important factor in the therapeutic field for Parkinson's disease. It will be interesting to see if supplementing taurine rescues the PD symptoms in animal models.

It will be important to determine the chemical structure of HOCD and to develop methods for detecting it in trace amounts. This will be necessary to confirm that HOCD is present in the midbrain of patients with PD. Furthermore, if HOCD or its metabolites can be detected in cerebrospinal fluid or blood, it might provide an opportunity for the early diagnosis of Parkinson's disease before motor symptoms appear.

The discovery of HOCD, nonetheless, opens multiple new approaches to study the etiology of Parkinson's disease at molecular as well as cellular levels. Hopefully, future research exploring these avenues will direct us to advanced therapies for curing Parkinson's disease.

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ABSTRACT

UNDERSTANDING THE MECHANISM OF OXIDATIVE STRESS GENERATION BY OXIDIZED DOPAMINE METABOLITES: IMPLICATIONS IN PARKINSON'S DISEASE

by

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Oxidation of dopamine to toxic metabolites is considered to be one of the prime factors involved in the death of dopaminergic neurons in Parkinson's disease. Some dopamine oxidation products have the capability to redox cycle in the presence of molecular oxygen, further contributing to oxidative stress. Therefore, our aim here was to study the redox cycling of dopamine oxidized metabolites and elucidate the underlying mechanism by which they cause oxidative stress.

Redox reactions involve transfer of one or more electrons between two compounds resulting in either oxidation or reduction. In redox cycling, a compound undergoes alternate oxidation and reduction, transferring electrons from a reductant to molecular oxygen. Therefore, we began by investigating different modes of redox cycling by measuring the rate of oxygen consumption using a Clark-type oxygen electrode in the presence of different reductants. We compared chemically synthesized redox cyclers such as menadione, 6-hydroxydopamine (6-OHDA), 3-methyl-5-anilino-1,2-benzoquinone (3-MAQ) and 9,10-phenanthrenequinone, using ascorbic acid and dithiothreitol (DTT) as reductants. Addition of superoxide dismutase diminished DTT-

dependent redox cycling activity (except in the case of menadione) but had no effect on the ascorbate-dependent redox cycling activity. This suggests that DTT drives a two-electron reduction whereas ascorbate causes a one-electron reduction. NADH-dependent redox cycling mediated by mitochondria was also studied using 3-MAQ. This mitochondrially mediated redox cycling activity was inhibited by mersalyl acid, thereby suggesting the involvement of the outer-mitochondrial membrane protein, NADH-dependent cytochrome b5 reductase, in the redox cycling mechanism.

We identified hypochlorite-oxidized cysteinyl-dopamine (HOCD) as a redox cycling product and a potential candidate for dopaminergic neuron toxicity in the progression of Parkinson's disease. The dopamine oxidation product cysteinyl-dopamine has attracted attention as a contributor to the death of dopaminergic neurons in Parkinson's disease. Treatment of cysteinyl-dopamine with hypochlorite yields an even more cytotoxic product. This product, HOCD, has potent redox-cycling activity and initiates production of superoxide in PC12 cells. Taurine, which scavenges hypochlorite, protects PC12 cells from cysteinyl-dopamine but not from HOCD, suggesting that HOCD, not cysteinyl-dopamine itself, is toxic. Furthermore, rotenone, which enhances expression of the hypochlorite-producing enzyme myeloperoxidase, increases the cytotoxicity of cysteinyl-dopamine but not of HOCD. This suggests that dopamine oxidation to cysteinyl-dopamine followed by hypochlorite-dependent conversion to a cytotoxic redox-cycling product HOCD, leads to the generation of reactive oxygen species and oxidative stress and may contribute to the death of dopaminergic neurons.

Our findings of HOCD toxicity in PC12 cells was followed by our study to determine the mode of cell death. The morphological changes in the cell such as membrane

blebbing and appearance of biochemical markers such as cleaved poly-ADP ribose polymerase and active caspase-9 suggested cell death by apoptosis. Moreover, increased expression of tumor suppressor protein p53, indicated mitochondrial mediated apoptotic cell death. Our observations have raised an unappreciated possibility that may link dopamine oxidation, microglial inflammation, oxidative stress and the rotenone model of Parkinson's disease. Furthermore, it offers a promising new approach in the search for a therapeutic cure for Parkinson's disease.

AUTOBIOGRAPHICAL STATEMENT

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EDUCATION

2010 – 2017   PhD in Biological Sciences, Wayne State University, Detroit, Michigan, USA.

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PUBLICATIONS

- **Mehta, N. J**., Asmaro, K., Hermiz, D. J., Njus, M. M., Saleh, A. H., Beningo, K. A., and Njus, D. (2016) Hypochlorite converts cysteinyl-dopamine into a cytotoxic product: A possible factor in Parkinson's Disease. *Free Radical Biology and Medicine* **101**, 44-52.
- **Mehta, N. J**., and Vaidya, V. K. (2010) Application of chromium resistant organisms in bioremediation. *Journal of Industrial Pollution Control* **1**.
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- **Mehta, N. J**., and Vaidya, V. K. (2009) Co-Existence of heavy metal and antibiotic resistance in bacteria isolated from polluted environment. *Bionano Frontier* **2(2).**

ORAL PRESENTATIONS

- Presentation titled, "Dopamine, Hypochlorite and Parkinson's Disease" at 44th Annual Meeting of the Society for Neuroscience, Washington D.C., November 2014.
- Presentation titled, "Hypochlorite converts dopamine oxidation products into redox-cycling compounds" at Annual Biological Sciences Departmental Retreat, Cranbrook Institute of Science, Bloomfield Hills, Michigan, October 2013.

POSTER AWARDS

- Annual Biological Sciences Departmental Retreat, Cranbrook Institute of Science, Bloomfield Hills, Michigan, October 2015, 2014 and 2012 **(Winner, 2nd place)**.
- Graduate Student Research Day, Wayne State University, Detroit, Michigan, September 2014. **(Winner, 1st place)**.