LEVODOPA DRUG INDUCED ALTERATION OF THIOL HOMEOSTASIS IN MODEL NEURONS ACTIVATES APOPTOSIS SIGNALING KINASE 1: IMPLICATIONS FOR THE TREATMENT OF PARKINSON’S DISEASE

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Dedication

This thesis is dedicated to my grandparents, Lena and Vincent Bordenca. Both strongly encouraged the maintenance of close, loving families and the importance of education.
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Abbreviations:

Aβ: Amyloid protein β
AD: Alzheimer’s Disease
ARE: Antioxidant response element
ASK1: Apoptosis signaling kinase 1
ATCC: American Type Culture Collection
AU: Absorbance Units
BME: β-mercaptoethanol
BSA: Bovine serum albumin
[3H]-BSA-SSG: S-carboxymethyl-Bovine serum albumin -NH2-CO-CH2-CH2-S-SG-[3H]
BSO: Buthionine sulfoximine
BV-2: Immortalized mouse microglial cell line
CNS: Central nervous system
CSSG: Cysteinyl-S-S-glutathione mixed disulfide
Cys: Cysteine
DA: Dopamine
Daxx: Death associated protein 6
DJ-1: A protein implicated in Parkinson’s disease that functions as an antioxidant, transcriptional co-activator, and molecular chaperone
DOPAC: 3,4-dihydroxyphenylacetic acid
DSS: Disuccinimidyl suberate
DTNB: 5′,5′-dithiobis-(2-nitrobenzoic acid), Ellman’s reagent
DTT: Dithiolothreitol
E1: Ubiquitin activating enzyme E1
ECL: Enhanced chemiluminescence
EDTA: Ethylenediaminetetraacetic acid
ELLDOPA: Early versus late levodopa clinical trial
ER: Endoplasmic reticulum
ERKS: Extracellular signal regulating protein kinases
FAD: Flavin adenine dinucleotide
GR: Glutathione Disulfide Reductase
Grx1: Glutaredoxin 1
Grx2: Glutaredoxin 2
GSH: Glutathione
GSSG: Glutathione disulfide
HEDS: Hydroxyethyl disulfide
6-OHDA: 6-hydroxydopamine
HSP: Heat shock protein
IgG: Immunoglobulin G
IL: Interleukin
IP: Immunoprecipitation
IκB: Inhibitor of NFκB
IKK: IκB kinase
IRE: Inositol requiring enzyme
JNK: cJun N terminal kinase
L-DOPA: L-3,4-dihydroxyphenylalanine
LN18: Immortalized human microglial cells
LNCaP: Lymph node prostate carcinoma cell
LPS: Lipopolysaccharide
MAPK: Mitogen activated protein kinase
MAPKKK: Mitogen activated protein kinase kinase kinase
MEKK: MAPK/ERK kinase kinase
MEM: Minimal essential media
MLK: Mixed lineage kinase
MN9D: Murine mesencephalic neurons 9D
MPP+: 1-methyl-4-phenylpyridinium
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS/MS: Tandem mass spectrometry
mtDNA: Mitochondrial DNA
MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH: Nicotinamide adenine dinucleotide phosphate
NF-1: Nuclear factor-1
NGF: Nerve growth factor
NFkB: Nuclear factor kappa B
NO: Nitric oxide
NOS: Nitric oxide synthase
Nrf2: Nuclear factor-E2-related factor 2
p38: p38 MAPK
PBS: Phosphate buffered saline
PC12: Immortalized pheochromocytoma cells

PCR: Polymerase chain reaction

PD: Parkinson’s disease

Prx1: Peroxiredoxin 1

P-SSG: Protein-SSG, protein glutathione mixed disulfide (glutathionylated protein)

Protein-SNO: S-nitrosylated protein

PTP1B: Protein tyrosine phosphatase 1B

PVDF: Polyvinyl difluoride

RIPA: Radio immuno precipitation assay

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RT: Room temperature

SB202190: Chemical inhibitor of p38 (Sigma)

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SHSY5Y: Immortalized dopaminergic neuroblastoma cells

siRNA: Short interfering RNA

SOD: Superoxide dismutase

SP600125: Chemical inhibitor of JNK (Sigma)

TCA: Trichloroacetic acid

TDOR: Thiol Disulfide Oxidoreductase

TGR: Testes specific isozyme of TR

TNFα: Tumor necrosis factor α
TRAF: TNF receptor associated factor

Trx1: Thioredoxin 1

Trx2: Thioredoxin 2

TR: Thioredoxin Reductase

UPDRS: Unified Parkinson’s disease rating scale
Levodopa drug Induced Alteration of Thiol Homeostasis in Model Neurons
Activates Apoptosis Signaling Kinase 1: Implications for the Treatment of Parkinson’s Disease

Abstract

By

ELIZABETH ANN SABENS

Parkinson’s disease (PD), the second most common neurodegenerative disease, is characterized by dopaminergic neuron loss, increased oxidative stress, mitochondrial dysfunction, and protein aggregation. Treatment of PD involves chronic administration of Levodopa (L-DOPA) which paradoxically induces cell death in cellular models of PD. We hypothesized that L-DOPA-induced cell death occurs due to increased oxidative stress disrupting sulfhydryl homeostasis through modification of homeostatic enzymes, glutaredoxin (Grx), GSSG reductase (GR), thioredoxin (Trx), and thioredoxin reductase (TR). Indeed, L-DOPA inhibited Grx in a dose-dependent fashion; however, its content was unaffected. GR activity was not altered. L-DOPA treatments also led to decreased activities of Trx and TR, usually concomitant with diminution of their cellular levels. Experiments involving treatment of the isolated enzymes with oxidized L-DOPA
established that only Grx is inactivated in a time- and concentration-dependent fashion, corresponding to irreversible adduction of dopaquinone to the active site cysteine (Cys-22). Furthermore, selective knockdown of Grx or chemical inhibition of TR resulted in increased apoptosis, documenting the neuroblastoma cell’s dependence on both the Grx and Trx systems for survival. In order to further elucidate the mechanism(s) of L-DOPA induced cell death, our studies focused on cell fate pathways regulated by both Grx and Trx. NFκB, normally a prosurvival transcription factor, is regulated by both Grx and Trx where oxidation of various proteins within its activation pathway results in decreased function. Apoptosis signaling kinase 1 (ASK1), a pro-apoptotic MAPKKK, is activated by oxidative stress leading to phosphorylation of downstream MAPKs, p38 and JNK, and initiation of apoptosis. Consistent with this mechanism, L-DOPA treatment of SHSY5Y cells leads to increased phosphorylation of the downstream ASK1 targets, JNK and p38. Furthermore, inhibition of JNK and p38 activity is sufficient to nearly abolish L-DOPA-induced cell death. Knockdown of ASK1 conferred near complete protection from L-DOPA-induced apoptosis, implicating the ASK1 pro-apoptotic pathway as the major effector of L-DOPA-induced cell death. These results elucidate a distinct mechanism of L-DOPA-induced apoptosis and provide the ability to develop new therapeutics aimed at preventing the unwanted side effect of L-DOPA in Parkinson’s disease therapy.
Chapter 1: Introduction and Background:

1.1 Parkinson’s Disease

Parkinson’s disease (PD), the second most common neurodegenerative disease, affects approximately 1% of the population over the age of 65, and 5% over the age of 85. Pathogenesis in PD patients is characterized by bradykinesia, resting tremor, rigidity, and postural instability, and is due to loss of catecholaminergic neurons in the substantia nigra of the brain (P. F. Riedlerer 2004). PD, linked to genetic factors as well as environmental factors such as pesticides, occurs through a process involving increased oxidative stress, mitochondrial dysfunction, and protein aggregation (K. A. Maguire-Zeiss et al. 2005). Oxidative stress has been implicated in the pathogenesis of PD in many reports and has been attributed to increased dopamine turnover, deficient glutathione (GSH) content, and increased iron in the substantia nigra (C. W. Olanow, W. G. Tatton 1999). Importantly, GSH loss is thought to be one of the early changes associated with onset of PD (R. Franco, J. A. Cidlowski 2009), which could initiate the increased oxidative stress.

1.2 Sources of Reactive Oxygen and Nitrogen Species in Brain

The main source of reactive species within the brain is the mitochondrial electron transport chain. Because the brain requires approximately 20% of the total body respiration despite its relatively small mass, the amount of ROS produced through leakage from the electron transport chain is meaningful (M. A. Smith et al. 2000). Superoxide, a byproduct of the electron transport chain, is produced mainly at complex I and complex III. Also, on the outer membrane of the mitochondria lies monoamine
oxidase, which produces hydrogen peroxide as a byproduct of its oxidative deamination reactions (M. T. Lin, M. F. Beal 2006; P. H. Reddy 2007). This may be of particular importance in PD with increased dopamine turnover increasing the amount of hydrogen peroxide produced through its oxidative deamination by monoamine oxidase B (C. W. Olanow, W. G. Tatton 1999). Another source of ROS within the mitochondria occurs in the Krebs cycle, specifically through α-ketoglutarate dehydrogenase.

Other sources of oxidants arise from NADPH oxidase, which is present in astrocytes, microglia, and neurons. Flavoproteins and other oxidases form hydrogen peroxide, contributing to the overall oxidant content within the cell body. Also, inflammatory cells present within the brain are sources of reactive nitrogen species (RNS) and reactive oxygen species (ROS). Inhibition of nitric oxide synthase (NOS) has been shown to be neuroprotective, implicating NOS in the production of radicals which can produce peroxynitrite and hydrogen peroxide (H. Ischiropoulos, J. S. Beckman 2003).

During neurodegenerative disease, advanced age is often a characteristic of the majority of patients. Increasing age affects mitochondria by increasing the number of mtDNA mutations and increasing ROS production from the electron transport chain. The mutations may alter the accuracy of the electron transport chain resulting in increased production of ROS (S. Reddy et al. 2000). Furthermore, calcium fluxes are less controlled with diminished electron transport function, which creates a calcium overload leading to increased oxidative stress. Neurodegenerative CNS tissues show elevated calcium levels corroborating this hypothesis (M. P. Mattson 2007).
1.3 **Levodopa: Benefits and Potential Oxidative Side Effects:**

The primary therapeutic agent is Levodopa (L-DOPA), used at increasing doses as the disease progresses. L-DOPA, the precursor to dopamine, replenishes dopamine levels alleviating symptoms in patients. However, with disease progression, patients require higher and/or more frequent doses of L-DOPA to maintain a therapeutic effect. Although L-DOPA is the gold standard therapy in PD patients, studies examining its role in disease progression have been inconclusive warranting further examination (S. Fahn 2006; J. E. Ahlskog 2007). Indeed, clinical trials, although not finding progression of the movement disorder as determined by clinical movement scoring (UPDRS), show increased neurodegeneration through neuroimaging (S. Fahn et al. 2004). Furthermore, in cell culture models, exposing cells to increasingly higher concentrations of L-DOPA has been shown to lead to cell death of cultured catecholaminergic cells as a result of oxidative stress (G. Walkinshaw, C. M. Waters 1995). Excess dopamine can cause oxidative stress through its metabolism whereby it is readily oxidized to dopamine quinone along with reactive oxygen species (ROS), superoxide and hydrogen peroxide. Also, dopaquinone can irreversibly conjugate to cysteine residues on proteins, forming S-cysteinyldopamine adducts, as a second mechanism of altering protein function (K. A. Maguire-Zeiss et al. 2005). It is conceivable that chronic L-DOPA treatment could exacerbate the disease state by increasing the oxidative stress placed upon dopaminergic neurons especially under reduced antioxidant capacity as described for PD patients (K. A. Maguire-Zeiss et al. 2005). Excess heavy metal, such as iron or manganese, can also react with L-DOPA.
L-DOPA is oxidized to semiquinone and quinone forms, releasing superoxide. Also, L-DOPA undergoes a decarboxylation reaction by L-amino acid decarboxylase to form dopamine. Dopamine can undergo similar oxidations to L-DOPA forming both semiquinone and quinone species. Formation of these oxidized products results in the release of superoxide. Lastly, dopamine is degraded by monoamine oxidase forming 3,4-dihydroxyphenylacetic acid (DOPAC). Formation of this degradation product releases superoxide. Also, DOPAC can similarly form the reactive quinone species as shown at the bottom of the figure.
to enhance its toxicity in vitro (R. Migheli et al. 1999). In rodents, it has been documented that L-DOPA treatment results in increased lipid peroxidation, a characteristic sign of oxidative stress (C. W. Olanow, W. G. Tatton 1999). Oxidation of L-DOPA is displayed in Scheme 1-1 (p 6).

1.4 Cellular Oxidant Defense Mechanisms:

Antioxidant defense inside cells is dependent in large part on sulfur-containing amino acids in proteins and non-protein cofactors. Enzymatic scavenging of reactive oxygen species is afforded through superoxide dismutase (SOD), catalase, peroxiredoxins (Prx), and glutathione peroxidase. These enzymatic systems function to diminish the ROS load on the cell by reducing superoxides, hydrogen peroxides, and lipid hydroperoxides.

Nonenzymatic reduction of ROS occurs mainly through interaction with the abundant non-protein tripeptide, glutathione (GSH). The sulfhydryl chemistry of both GSH and of protein cysteine residues is critical for many cellular functions including cell growth, cell death, cell survival, and cell signaling. Since sulfhydryl redox status plays such an important role in the regulation of cell function, it is easy to perceive how perturbations of the antioxidant system including GSH and sulfur amino acids can be linked to abnormal cell functioning, aging, and many diseases including neurodegenerative diseases, arthritis, diabetes, cardiovascular disorders, AIDS, and cancer (K. Hirota et al. 2002). Oxidative stimuli, generated either through normal cell signaling, or overt oxidative stress, is associated with many disease conditions. This oxidative stress is associated with modifications of the sulfur-containing amino acids, cysteine (Cys) and methionine (Met), with subsequent effects on protein function and intracellular
homeostasis. Within cells, oxidants alter free cysteine residues in either a reversible or irreversible fashion. Irreversible oxidations include sulfinic (RSO₂H) and sulfonic (RSO₃H) acid formation, usually leading to increased protein degradation. In contrast, reversible thiol modifications, mainly through formation of protein mixed disulfides with GSH, i.e., protein-glutathionylation, as well as other reversible thiol modifications including intramolecular disulfides, intermolecular disulfides, and protein sulfenic acids are thought to protect proteins from degradation, allowing for a return to normal function after the oxidant challenge has subsided.

Alternatively, reactive nitrogen species (RNS) can modify protein thiols by nitric oxide (NO). Formation of S-nitrosylated proteins (Protein-SNO) occurs through this modification. Furthermore, ROS and RNS can form peroxynitrite which can mediate Cys modification. Protein-Cys modification via S-nitrosylation has been reported for several proteins including Trx and procaspase 3 (D. R. Arnelle, J. S. Stamler 1995; J. B. Mannick 2007) (Scheme 1-2, p 9).

Oxidative modifications of Cys sulfhydryl moieties must be reduced to restore protein function, and this homeostatic process is catalyzed by enzymes. An important class of enzymes called thiol-disulfide oxidoreductases (TDORs) is involved in catalyzing thiol-disulfide exchange reactions occurring on the cysteine-thiol groups. The two principal TDORs that are involved in sulfhydryl homeostasis within cellular systems are thioredoxin (Trx) and glutaredoxin (Grx), which catalyze the reduction of disulfide bonds in both protein and non-protein substrates (D. M. Ziegler 1985; J. J. Mieyal et al. 1995; J. J. Mieyal et al. 2008; E. A. Sabens, J. J. Mieyal 2009). Each of these TDOR
Scheme 1-2: Sulphydryl Homeostasis and Regulation: Oxidation and Regeneration of Vital Proteins.

Oxidative stimulus leads to multiple types of sulfhydryl modifications, including intramolecular disulfides, intermolecular disulfides, glutathionyl-protein mixed disulfides, protein sulfenic acids, and nitrosylated proteins. The latter two readily form protein-SSG mixed disulfides upon interaction with glutathione. Protein sulfenic acids also can be further oxidized to the irreversible protein sulfinic and sulfonic acids.
**Scheme 1-3:** Oxidation of Protein Thiols and Reduction by the Trx and Grx Systems

Selective activities of the thioredoxin (Trx) and glutaredoxin (Grx) systems. Grx selectively reduces glutathionylated proteins (Protein-SSG), whereas Trx is able to reduce intermolecular disulfides and intramolecular disulfides.
enzyme types has displayed substrate selectivities and redox potentials indicative of distinct physiological functions. Trx effectively reduces intramolecular and intermolecular disulfide bonds as well as sulfenic acids (C. Jacob et al. 2006) and protein-SNO (M. Benhar et al. 2009). Grx specifically reduces protein-glutathione mixed disulfides (Protein-SSG) (S. A. Gravina, J. J. Mieyal 1993; J. J. Mieyal et al. 1995).

**Scheme 1-3** (p 10) illustrates the catalytic cycles for both Grx and Trx which are discussed below in their respective sections on mechanism of action. The special characteristics of the thioredoxin and glutaredoxin enzyme systems suggest different but complimentary functions for them, likely acting synergistically to maintain the thiol status in various types of cells.

TDORs have a characteristic active site motif of four amino acids, Cys-Xaa-Xaa-Cys, and they exhibit a similar three dimensional structure referred to as the “thioredoxin fold” which is comprised of 5 β sheets enfolded within 4 α helices. The TDORs featured here, Grx and Trx, are small (approximately 12 kDa), heat stable proteins. Mechanistically, Grx and Trx function differently in their primary catalytic reactions, with Grx utilizing only one of the active site cysteines to effect specific reduction of glutathione-containing mixed disulfides (*i.e.* a monothiol mechanism) (Y. Yang *et al.* 1998; M. D. Shelton *et al.* 2005; M. M. Gallogly *et al.* 2009), whereas Trx uses both cysteines in the active site to reduce intramolecular disulfides (*i.e.* a dithiol mechanism) (C. Johansson *et al.* 2004). Grx and Trx are recycled from their oxidized forms by separate coupling enzyme systems, both dependent on the cofactor NADPH (**Scheme 1-4**, p 12).
Scheme 1-4: Reaction Mechanism of Grx and Trx

(A) Monothiol mechanism of glutaredoxin (Grx), showing specificity for glutathionylated substrates. Efficient catalysis cycles around intermediate formation of a covalent enzyme-glutathione mixed disulfide intermediate (Grx-SSG) not involving the second cys residue at the active site. Nonglutathione-containing disulfides must first react with GSH to form the actual substrate for Grx (upper left). Grx can form an intramolecular disulfide, but this detracts from catalysis and must react with GSH to return to the effective catalytic cycle. Grx-SSG is reduced by GSH, forming the dimeric disulfide, GSSG. GSSG is subsequently reduced by GSSG reductase (GR) and NADPH. (B) Thioredoxin (Trx) dithiol mechanism shows preference for reduction of inter- or intramolecular disulfides. The intramolecular disulfide form of Trx is reduced by thioredoxin reductase (TR) and NADPH. Scheme adapted from (E. A. Sabens, J. J. Mieyal 2009)
1.5 General Characteristics of Glutaredoxins:

The family of enzymes called glutaredoxins (Grx), also known as thioltransferases according to the reactions that they catalyze, is part of the broader TDOR superfamily. As indicated above, the distinguishing reaction catalyzed by the Grx enzymes is reduction of protein-glutathione mixed disulfides (protein-SSG) via thiol-disulfide exchange (i.e. thiol transfer). S-glutathionylation of specific Cys residues on particular proteins occurs as a normal physiological response to extracellular effectors (hormones, cytokines, etc.). This modification allows propagation of cell signaling cascades initiated by reactive oxygen species (ROS) or reactive nitrogen species (RNS) acting as second messengers. Protein-SSG formation also serves as a homeostatic response under oxidative stress conditions to prevent irreversible oxidation of the thiol moieties of cysteine residues on proteins (Scheme 1-2, p 9). Analogous to kinase signaling pathways where the extent of phosphorylation is regulated by phosphatases (enzymes that remove a phosphate group from phosphorylated serine, threonine, or tyrosine residues on proteins) glutaredoxins deglutathionylate proteins, specifically and efficiently removing the glutathione and restoring the reduced Cys-SH residue (J. J. Mieyal et al. 1995; P. Klatt, S. Lamas 2000; M. D. Shelton et al. 2005; C. H. Lillig, A. Holmgren 2007).

Mammalian Grxs include Grx1, Grx2, and Grx5 (E. A. Sabens, J. J. Mieyal 2009); Grx1 and Grx2 have been well characterized (see below). Grx5 containing one thiol in its active site is reported to function in FeS cluster biosynthesis and is required for yeast survival (R. A. Wingert et al. 2005). A mutation in Grx5 in humans results in diminished FeS clusters in red blood cells (C. Camaschella et al. 2007). More isoforms
of Grx (Grx1-7) have been identified in *Escherichia coli* (*E. Coli*) and yeast. However, not all glutaredoxin isoforms function as efficient deglutathionylating enzymes (M. M. Gallogly *et al.* 2009).

Grx1 contains a “CXXC-motif” at its active site with the specific sequence of CPYC; Grx1 also contains 3 nonactive site Cys (Cys 7, 78, 82) (J. J. Mieyal *et al.* 1991b). The Grx1 isoform is the better characterized isoform in mammalian systems, present in micromolar levels in mammalian cells (D. W. Starke *et al.* 1997). Grx1 is primarily localized to the cytosol, and it has also recently been shown to exist in the intermembrane space of mitochondria (H. V. Pai *et al.* 2007; M. M. Gallogly, J. J. Mieyal 2007; M. M. Gallogly *et al.* 2009); however, the specific functions of Grx1 in the mitochondria have yet to be elucidated. Localization of Grx1 in the nucleus of particular types of cells has been reported previously in immunocytochemical studies, specifically in the nuclei of calf thymus cells (B. Rozell *et al.* 1993), and within the stromal and epithelial cells of the human cervix (L. Sahlin *et al.* 2000; A. Stavreus-Evers *et al.* 2002; J. Lysell *et al.* 2003), however further studies including nuclear isolation are needed to verify localization and to determine whether Grx1 translocates to the nucleus under certain conditions in other types of cells.

A second mammalian glutaredoxin (Grx2), containing 164 amino acids with a molecular weight of 18kDa was discovered by an unusual database search which focused on particular domains rather than overall sequence similarity (E. A. Sabens, J. J. Mieyal 2009). Grx2 displays only about 30% sequence homology to Grx1 (M. Lundberg *et al.* 2001; V. N. Gladyshev *et al.* 2001; C. H. Lillig, A. Holmgren 2007). Grx2 has been shown to be localized to the mitochondrial matrix (V. N. Gladyshev *et al.* 2001; H. V. Pai
et al. 2007), bound to Fe-S clusters (C. Johansson et al. 2004; C. H. Lillig et al. 2005). Some reports show a second isoform of Grx2, Grx2b, to localize to the nucleus or perinuclear region (M. Lundberg et al. 2001; B. J. Goldstein 2001; M. E. Lonn et al. 2007), however more studies will need to be performed to document nuclear localization.

1.6 General Characteristics of Thioredoxins:

Thioredoxin enzymes are characterized by a thioredoxin fold as well as the “CXXC” motif at the active site, specifically CGPC, and they are reported to selectively catalyze the reduction of intra- and intermolecular disulfides as well as protein sulfenic acids (B. Mannervik et al. 1983; S. A. Gravina, J. J. Mieyal 1993) and more recently protein-SNO (M. Benhar et al. 2009). There are many members of the Trx family in many biological systems; however, only two, Trx1 and Trx2, have been characterized in mammals. The well studied Trx1 system has been linked to cell signaling, cell growth, promotion of cell survival, and initiation of an immune response (G. Powis et al. 2000; A. Burke-Gaffney et al. 2005).

Trx1, a 13 kDa protein, was initially discovered in E. Coli as an electron donor to ribonucleotide reductases (T. C. Laurent et al. 1964). Human Trx1, which like Grx is ubiquitously expressed, was initially identified under multiple names, including adult T cell leukemia-derived factor, early pregnancy factor, and interleukin-2 receptor inducing factor; it is a 105 amino acid protein with a molecular weight of 13kDa (S. Gromer et al. 2004). Mammalian Trx1 contains 3 non active site cysteines (Cys 62, Cys 69, and Cys 73) which are not found in bacterial Trx (G. Powis, W. R. Montfort 2001; A. Burke-
Upon oxidation of these Cys residues, Trx undergoes protein aggregation and loss of activity (A. Holmgren 1985).

Trx1 is mainly a cytosolic enzyme, playing critical roles in redox homeostasis as well as in cellular signaling. It has been shown to translocate to the nucleus under overexpression conditions or be exported from the cell under certain stimuli, including UV irradiation, as demonstrated by immunocytochemistry (H. Masutani et al. 1996; C. H. Lillig, A. Holmgren 2007).

The Trx1 knockout mouse is embryonic lethal, illustrating its importance for mammalian development and its necessity for supporting the activity of ribonucleotide reductases (M. Matsui et al. 1996). In contrast, the Grx1 knockout mouse is not embryonic lethal. Grx1 knockout does not impair development of the normal mouse (Y. S. Ho et al. 2007), nor does depletion of GSH interfere with DNA synthesis and ribonucleotide reductase activity in cell culture models (G. Spyrou, A. Holmgren 1996; Y. S. Ho et al. 2007). These observations strongly implicate Trx1 as the more important reducing enzyme for ribonucleotide reductase, distinguishing it from Grx1 which is more strongly implicated in other physiological functions (described below).

A second form of thioredoxin, Trx2, is localized to the mitochondria and contains an active site CGPC motif like Trx1. Unlike Trx1, Trx2 lacks any non-active site cysteine residues, and it contains a mitochondrial localization sequence accounting for its larger initial size of 18kDa (W. H. Watson et al. 2004). However, Trx2 isolated from mitochondria lacks the 60 amino acid mitochondrial import sequence, reflecting an intramitochondrial size of 12kDa, similar to that of cytosolic Trx1 and indicating
proteolytic cleavage of the import sequence within the mitochondria (W. H. Watson et al. 2004).

1.7 Glutaredoxin Mechanism of Action:

Grx, with GSH as co-substrate, functions to reduce protein-SSG mixed disulfides as shown in scheme 1-4 (p 12). Grx works via a monothiol mechanism through a selective double displacement reaction in which the nucleophilic attack is performed by the N-terminal active site Cys (Cys 22) of the CPYC motif, which exists as a thiolate anion due to its unusually low pKa of 3.5 (Y. F. Yang, W. W. Wells 1991; J. J. Mieyal et al. 1991b; W. W. Wells et al. 1993). The glutathionylated sulfur moiety of the protein-SSG is attacked by the thiolate anion of the enzyme (Grx-S'), forming the covalent enzyme intermediate (Grx-SSG) and releasing the reduced protein-SH as the first product. The second, rate determining step involves reduction of the Grx-SSG by GSH to produce glutathione disulfide (GSSG) as the second product, and recycle the reduced enzyme (Grx-S'). GSSG is subsequently reduced to GSH by GSSG reductase (GR) and NADPH. Also, in systematic kinetics studies Grx (a.k.a. thioltransferase) was shown to be specific for glutathione-containing disulfides as the first substrate (S. A. Gravina, J. J. Mieyal 1993; Y. Yang et al. 1998; C. A. Chrestensen et al. 2000; C. Johansson et al. 2004; S. C. Jao et al. 2006), and GSH is the preferred second substrate (U. Srinivasan et al. 1997) for the two step reaction. (details reviewed in (M. M. Gallogly et al. 2009)).

Mutagenesis studies that replace the second Cys at the active site (distal from the N-terminus, Cys 25) have supported the monothiol mechanism. When this Cys is replaced (e.g. by serine, C25S mutation of human Grx1), Grx1 retains its normal catalytic
function, and in fact becomes a better catalyst than the natural enzyme with both Cys residues at the active site (Y. Yang et al. 1998). This observation documents that the side reaction involving formation of the intramolecular disulfide form of the enzyme (C22-SS-C25) detracts from catalysis (Scheme 1-4, p 12).

The catalytic cycle for Grx-mediated reactions (Scheme 1-4, p 12) represent a nucleophilic double displacement mechanism in which Grx acts upon a glutathionylated substrate to form the Grx-SSG intermediate, which is subsequently recycled by reaction with GSH. This reaction scheme is documented by so-called ping-pong kinetics which give a characteristic parallel line pattern for the 1/V vs. 1/S plots at several fixed concentrations of the co-substrate. This kinetic behavior has been documented for both isozymes of mammalian glutaredoxin (Grx1 & Grx2) (S. A. Gravina, J. J. Mieyal 1993; J. J. Mieyal et al. 2008; M. M. Gallogly et al. 2008). If a non-glutathionylated precursor is tested as the first substrate, then the two-substrate kinetics pattern changes to an ordered mechanism with a double reciprocal plot displaying converging lines at the same point on the x-axis (i.e. identical apparent Km values), reflecting the requirement of the initial reaction of the precursor with GSH to form the actual glutathionylated substrate for the enzyme (J. J. Mieyal et al. 1991b; M. M. Gallogly et al. 2009).

Analogous to Grx1, Grx2 exhibits deglutathionylating activity for peptide and protein substrates, but its activity is approximately ten-fold lower than that of Grx1 (C. Johansson et al. 2004; C. Johansson et al. 2007; M. M. Gallogly, J. J. Mieyal 2007; M. M. Gallogly et al. 2008). Mutating the active site of Grx2 (CSYC) to mimic that of Grx1 (CPYC) partially enhances the Grx2 activity but Grx2 still remains less active than Grx1 (C. Johansson et al. 2004), indicating that other features of the two proteins contribute the
distinction in activity as described in (M. M. Gallogly et al. 2008). However, Grx2 activity within the cell may be further limited due to sequestration in Fe-S clusters where the Grx2 active site is masked (C. H. Lillig et al. 2005). It has recently been reported that treatment with oxidants (e.g. S-nitrosoglutathione) causes the release of Grx2 from these Fe-S clusters \textit{in vitro} allowing for activity to be reestablished (S. I. Hashemy et al. 2007). Hence, release of Grx2 from Fe-S clusters may represent a protective response to oxidative stress, thereby providing for thiol-disulfide homeostasis within the mitochondria.

Turnover of the Grx-SSG intermediate by GSH results in formation of GSSG, as shown in scheme 1-4 (p 12). GSSG reductase (GR) is the enzyme responsible for reducing GSSG to replenish GSH. GR is a ubiquitously distributed protein whose primary function is to maintain a high GSH:GSSG ratio in most intracellular regions, with the exception of the endoplasmic reticulum where a more oxidizing environment is maintained (C. Hwang et al. 1992). GR consists of two identical subunits as well as two bound FAD molecules. Each subunit contains 4 domains which include the FAD binding domain containing two redox active thiols, the NADPH binding domain, the interface domain, and the central domain (R. Untucht-Grau et al. 1981). The kinetics of the GR reaction implicates a \textit{ping pong} mechanism although a different mechanism involving a ternary complex of enzyme and substrates has been proposed for high GSSG concentrations (M. A. Vanoni et al. 1990). GR in the mitochondria allows for the reduction of GSSG to GSH maintaining the mitochondria in a reduced state.
1.8 Thioredoxin Mechanism of Action:

Thioredoxin, containing the active site CGPC, catalyzes reduction of inter- and intramolecular disulfides, as well as protein sulfinic acids via a two step mechanism involving intermediate formation of the intramolecular disulfide form of thioredoxin. This oxidized form of Trx is recycled by thioredoxin reductase (TR) and NADPH, without the participation of glutathione. Thus, the catalytic mechanisms for Trx and Grx are quite distinct. Both involve nucleophilic double displacement reactions where thiols act as the nucleophiles, but the Trx intermediate is turned over directly by another enzyme, whereas the Grx intermediate is turned over by a second substrate (GSH) (Scheme 1-4, p 12). Trx acts on oxidized sulphhydryls effectively due to the lower pKa (6.8-7.0) of its N-terminal active site Cys which makes it more nucleophilic compared to the typical pKa of Cys (~8.5). Stabilization of the Cys-thiolate anion is attributed to the surrounding basic amino acids in the active site (G. B. Kallis, A. Holmgren 1980; A. Holmgren 1985). The best studied substrates for Trx are intramolecular disulfides such as the oxidized form of the ribonucleotide reductase enzyme, and accordingly, the best characterized assay for Trx involves reduction of the prototype intramolecular disulfide substrate insulin (M. Luthman, A. Holmgren 1982). Trx requires both active site Cys residues to effect reduction of disulfide bonds (dithiol mechanism), whereas Grx only requires the C-terminal Cys to be functional (monothiol mechanism, described above). Besides reversal of particular oxidized cysteine modifications, Trx also supports the reduction of methionine sulfoxides (oxidized methionine residues). The methionine sulfoxides are acted upon directly by methionine sulfoxide reductases (MSR-A, MSR-B), forming the intermediate MSR-intramolecular disulfide which is a typical substrate for
Trx. Trx coupled to TR recycles this intermediate back to its reduced, functional form, allowing for the continual reduction of methionine sulfoxides back to methionine (M. Antoine et al. 2003). Multiple reviews report Trx working through a ping pong mechanism in the reduction of disulfides. Although this is a reasonable interpretation, two substrate kinetics studies documenting this mechanism were not included in the original cited studies (A. Holmgren 1979). Accordingly, oxidized Trx, with its active site as an intramolecular disulfide, is reverted back to the reduced dithiol form by TR (Scheme 1-4, p 12) (G. Powis, W. R. Montfort 2001).

Besides serving as the coupling enzyme for turnover of Trx-disulfide, TR also is able to reduce small molecules, including selenite (M. Bjornstedt et al. 1995), GS-Se-SG (M. Bjornstedt et al. 1995), alloxan (A. Holmgren, C. Lyckeborg 1980), and vitamin K (A. Holmgren 1985). TR is a FAD-containing enzyme comprised of two subunits held together nonconvalently. Currently three mammalian TR enzymes are known, TR1, the cytosolic form, TR2, the mitochondrial form, and TGR, a testis-specific isoenzyme which is a fusion protein containing multiple TDOR domains. Mammalian TR enzymes are large selenoproteins consisting of two 55 kDa subunits (S. Gromer et al. 2004) containing a penultimate selenocysteine on the C-terminus, allowing for substrate specificity not seen in the bacterial system (C. H. Lillig, A. Holmgren 2007).

1.9 Cellular Functions of Grx:

Protein glutathionylation is a reversible posttranslational modification on cysteine residues that occurs under oxidative stress or in response to an oxidative stimulus and it may result in alteration of protein function. For example, S-glutathionylation leads to
inhibition of protein tyrosine phosphatase-1B (PTP1B) (W. C. Barrett et al. 1999a; W. C. Barrett et al. 1999b), but formation of hRas-SSG leads to activation (D. R. Pimentel et al. 2006). Such regulation of cellular functions via reversible S-glutathionylation of Cys residues on specific proteins has evolved recently as an area of intense scientific investigation (reviewed in (P. Klatt, S. Lamas 2000; M. D. Shelton et al. 2005; P. Ghezzi 2005; J. J. Mieyal et al. 2008)). Although studies of isolated proteins and proteomic approaches have identified a large number of proteins as potentially being regulated via S-glutathionylation, only a few protein-SSG adducts have been documented as regulatory intermediates in a cellular context. As a guide to studying the literature on reversible glutathionylation as a regulatory mechanism in redox signal transduction, our research group has proposed five criteria (M. D. Shelton et al. 2005). Namely, S-glutathionylation in a regulatory context has the following characteristics: (1.) alters the function of the modified protein, (2.) occurs in intact cells as a response to a physiological stimulus and elicits a physiological response, (3.) occurs at normal GSH:GSSG ratios, (4.) occurs in a rapid and efficient manner, and (5.) is reversed in a rapid and efficient manner. Criterion #5 is fulfilled by the action of glutaredoxin. Thus, the chief function of Grx is to catalyze deglutathionylation, and manipulation of its content in cells has been used effectively to document regulatory pathways that involve S-glutathionylated intermediates (J. J. Mieyal et al. 2008). S-glutathionylation and Grx-mediated deglutathionylation has been implicated in the regulation of a number of proteins including cytosolic proteins such as Ras (T. Adachi et al. 2004) and actin (J. Wang et al. 2003), transcription factors such as NF-1 (S. Bandyopadhyay et al. 1998) and NFκB (E. Pineda-Molina et al. 2001; S. Qanungo et al. 2004; S. Qanungo et al. 2007; M. D. Shelton et al. 2007), apoptosis
mediators such as Akt (F. H. Pham et al. 2000; H. Murata et al. 2003) and cJun (P. Klatt et al. 1999b). This post translational thiol modification can also result in signaling events including cell proliferation (M. Kanda et al. 2006), cytoskeletal organization (J. Wang et al. 2001; J. Wang et al. 2003), transcription (E. Pineda-Molina et al. 2001; N. L. Reynaert et al. 2006; M. D. Shelton et al. 2007), and protein synthesis (T. Adachi et al. 2004). Changes in the S-glutathionylation status of proteins occurring at normal GSH:GSSG levels has been implicated also in many diseases including cancer, cardiovascular disease, diabetic retinopathy, and neurodegenerative diseases (D. Giustarini et al. 2004; J. J. Mieyal et al. 2008).

1.10 Cellular Functions of Trx:

Trx has many roles within the cellular environment including acting as a cofactor, a general antioxidant, an anti-apoptotic factor, and as a regulator of transcription factors. Since knowledge of Trx preceded Grx, and the extent of study of Trx exceeds that of Grx, many redox events have been interpreted to be regulated by Trx that may also or instead be regulated by Grx. Additionally, as a cellular safeguard there may be redundancy of regulation of various targets by Grx and Trx, each acting by distinct mechanisms. Trx has multiple roles within the cell. Trx can act as a cofactor for ribonucleotide reductase turnover (G. Powis et al. 2000), and it is hypothesized that this function rationalizes the embryonic lethality of the Trx1 knockout mouse. Trx also acts as an antioxidant through reduction of reactive oxygen scavenging enzymes reducing peroxiredoxins and methionine sulfoxide reductase (G. Powis, W. R. Montfort 2001; R. J. Arai et al. 2006). Trx regulates transcription through reduction of a number of transcription factors

Furthermore, Trx has also been implicated in the cellular defense against apoptosis induced by a variety of agents including dexamethasone, staurosporine, and etoposide. Indeed, multiple types of cancers have elevated levels of the Trx protein, and Trx overexpression is associated with cell growth and evasion of apoptosis, rendering cancer cells resistant to common chemotherapeutic agents. Importantly, the anti-apoptotic function of Trx was documented in studies where it was overexpressed in the cells, so caution is warranted in interpreting physiological relevance. These findings may reflect a general antioxidant function of Trx rather than a specific mode of inhibition of apoptosis since no molecular mechanism was described (G. Powis, W. R. Montfort 2001).

1.11 Reversible Sulfhydryl Oxidation and Disease:

Sulfhydryl modification occurs when cells are subjected to oxidative stress creating an oxidizing environment. However, only severe oxidative challenges would alter the GSH: GSSG ratio sufficiently so that GSSG concentration would be high enough to promote glutathionylation by thiol-disulfide exchange (i.e. not thermodynamically favored). Thus, alternative mechanisms of protein-SSG formation are more likely (M. D. Shelton et al. 2005;M. M. Gallogly, J. J. Mieyal 2007). Currently, little is known about how S-glutathionylation occurs. S-glutathionylation could occur via
reaction of oxidized glutathionyl radical (GSOH or GS\(^+\)) with free sulfhydryls and/or via enzyme catalyzed reactions, e.g. catalysis by glutathione-S-transferase or glutaredoxin (M. M. Gallogly, J. J. Mieyal 2007).

Maintenance of an active sulfhydryl repair system, mainly through the TDORs, Grx and Trx, is critical to prevent irreversible damage. However, in many disease states alterations in activity of either of these enzymes (by downregulation or direct inhibition of active site sulfhydryl modification) can lead to deleterious outcomes including enzyme inhibition, protein aggregation, and commitment to apoptosis through inhibition of transcription factors or alterations in signaling pathways. Protein modification via sulfhydryl oxidation has been associated with numerous diseases that involve oxidative stress including AIDS, Friedrich’s Ataxia, Type I and II diabetes, and ischemia reperfusion injury (D. Giustarini et al. 2004). The emerging roles of Grx and Trx in neurodegenerative diseases are described further below.

### 1.12 Glutaredoxin and Neurodegeneration

Glutaredoxin has been shown to be expressed throughout the brain where its high activity in the brain is similar to that found in liver (~0.05 Units/mg) (S. Balijepalli et al. 1999; J. Ehrhart et al. 2002). Grx2 was also recently shown to be active in both mouse and human brain (S. Karunakaran et al. 2007b). Despite Grx activity in brain, it has not been extensively studied in many neurodegenerative disease models. PD is modeled in mice with the treatment of a specific complex I inhibitor, 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP). Upon treatment with MPTP, mice show an increase in Grx activity followed by a restoration of complex I activity implicating the necessity of Grx
for Complex I function. Accordingly, diminution of Grx activity by antisense to Grx1 inhibited recovery of complex I activity (R. S. Kenchappa, V. Ravindranath 2003). Immunohistochemical studies performed on AD brains showed increased expression of Grx1 in healthy neurons; however, in neurons showing signs of neurodegeneration, Grx1 immunoreactivity was decreased compared to neurons from control brains, as measured in the frontal cortex and hippocampal regions (S. Akterin et al. 2006). As discussed previously, Grx is the specific and efficient de-glutathionylase catalyzing redox regulation of multiple proteins through reversible S-glutathionylation.

Alternatively, Grx has been shown to act as a negative regulator of activation of apoptosis signaling kinase 1 (ASK1) through its ability to bind to its C-terminus (J. J. Song, Y. J. Lee 2003). ASK1 has been shown to be associated with dopaminergic cell death in Parkinson’s disease models (see citations below). Under low glucose conditions, ASK1 activates a signaling cascade that includes activation of MEKK4/6 and JNK in MCF-7 cells (J. J. Song, Y. J. Lee 2003). This pathway is also activated in the human neuroblastoma cell line SHSY5Y upon treatment with 6-hydroxydopamine leading to cell death (M. Ouyang, X. Shen 2006). ASK1 has also been shown to be activated in mouse models of Parkinson’s disease upon treatment with MPTP. This activation is inhibited with addition of α-lipoic acid, a thiol antioxidant (S. Karunakaran et al. 2007a). ASK1 has been shown to be regulated by Grx in various cell lines where excess oxidative stimuli, including H$_2$O$_2$ or glucose deprivation, leads to dissociation of Grx from ASK1 and allows for phosphorylation and activation of the ASK1 apoptotic cascade (see below).

1.13 Thioredoxin and Neurodegenerative Disease:

Trx has been shown to have neuroprotective effects in the nervous system. Overexpression of Trx in murine brain leads to increased life span (A. Mitsui et al. 2002), resistance to focal ischemia (Y. Takagi et al. 1999), and resistance to excitotoxic stress as compared to wild type littermates (Y. Takagi et al. 2000). Trx1 protein and mRNA are upregulated in PC12 cells treated with nerve growth factor (NGF), and are associated with neurite formation. Furthermore, Trx binds ASK1 prohibiting its downstream signaling and initiation of apoptosis (M. Saitoh et al. 1998), described below. Trx may be decreased in Alzheimer’s disease; however, this interpretation is tentative due to a limited sample size (M. Asahina et al. 1998). Playing a general role as an antioxidant
could represent the importance of Trx to various oxidant stress diseases of the brain including Alzheimer’s disease and Parkinson’s disease.

1.14 Involvement of NFκB and MAPK Signaling Pathways in PD

Regulation of cell fate pathways have been shown in multiple contexts to be influenced by both oxidative state and the thiol homeostatic enzymes, Grx and Trx. One pathway recognized as a pro-survival pathway, NFκB, is regulated by both Grx and Trx at various steps within its activation pathway (Scheme 1-5, p 31). NFκB is a transcription factor comprised of multiple subunits. NFκB normally exists in an inactivated state, sequestered in the cytoplasm via noncovalent binding to IκB. NFκB becomes activated upon release from IκB, facilitating rapid translocation to the nucleus where it interacts with target gene promoters (S. Hunot et al. 1997). IκB releases NFκB upon its phosphorylation by IκB kinase (IKK). NFκB can be activated by multiple stimuli (M. K. Meffert, D. Baltimore 2005), including oxidative stress which plays a major role in PD. Thus, NFκB has been shown to be increased in nuclei of dopaminergic neurons of postmortem PD patients by immunohistochemistry (S. Hunot et al. 1997). Despite the observed increase in NFκB protein content, its activity was not examined and the downstream effector molecules were not reported.

The NFκB pathway can be redox regulated on multiple levels including the p50 subunit of NFκB (E. Pineda-Molina et al. 2001) and upstream at IKK (N. L. Reynaert et al. 2006). Also, exogenous Grx2 was reported to promote activation of NFκB and cell survival (D. Daily et al. 2001). However, localization and mechanism of action of the added Grx2 was not characterized. In cell culture models, NFκB has been related to two
different outcomes, cell death and cell survival. After stimulation with either oxidized dopamine, 6-hydroxydopamine (6-OHDA) or the proteosome inhibitor, PSI, NFκB activation was shown to be antiapoptotic (G. Taglialatela et al. 1997; H. J. Lee et al. 2001). However, others have shown NFκB to be proapoptotic within the same cell line (PC12), with treatment of dopamine or 6-OHDA suggesting that type or duration of stimulus may change the outcome of NFκB activation (H. Panet et al. 2001; V. Tarabin, M. Schwaninger 2004). Also, Ghosh et al. reported NFκB activation upon MPTP treatment of mice to be proinflammatory, contributing to cell death (A. Ghosh et al. 2007). The NFκB regulated genes include both prosurvival factors (e.g., Bcl-2), and proapoptotic factors (e.g., p53, Fas), as well as other genes involved in dopamine production specifically tyrosine hydroxylase (S. C. Kumer, K. E. Vrana 1996). Redox regulation of the NFκB pathway by Grx1 allows for reversible changes in activity of this transcription factor under various oxidative conditions. The response of NFκB to L-DOPA treatment has not been characterized (see Chapter 3).

Redox regulation of this pathway occurs upstream of NFκB as well as directly on NFκB. Targets for redox regulation by Trx1 include regulation of release of NFκB from IκB and reduction of oxidized NFκB, allowing for DNA binding (reviewed in (H. Nakamura et al. 1997; J. Nordberg, E. S. Arner 2001)). Also, Grx1 regulates both DNA binding of NFκB (S. Qanungo et al. 2007) and activation of IKK, allowing for its phosphorylation of IκB (M. D. Shelton et al. 2009) (Scheme 1-5, p 31).
Table 1: Protein Glutathionylation Affects Cell Signaling in Neurodegeneration

Glutathionylation targets in neurodegenerative diseases. The table provides a listing of various proteins that have been implicated as targets of glutathionylation and corresponding inhibition of protein function within the context of oxidative stress associated with neurodegenerative diseases.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Oxidative Challenge to Induce Glutathionylation</th>
<th>Affect on Activity</th>
<th>Evidence in Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>Increased iron found in fibroblasts of patients</td>
<td>inhibitory</td>
<td>Friedreich's Ataxia</td>
<td>Pastore et al. 2003</td>
</tr>
<tr>
<td>GADPH</td>
<td>isolated fibroblasts from HD and AD patients</td>
<td>inhibitory</td>
<td>Alzheimer's Disease, Huntington's Disease</td>
<td>Mazzola and Sirover 2001</td>
</tr>
<tr>
<td>Complex I</td>
<td>increased GSSG</td>
<td>inhibitory</td>
<td>Parkinson's Disease</td>
<td>Beer et al. 2003</td>
</tr>
<tr>
<td>IDPm</td>
<td>MPTP in mice</td>
<td>inhibitory</td>
<td>Parkinson's Disease</td>
<td>Kil and Park 2005</td>
</tr>
<tr>
<td>E1 Ubiquitin Enzyme</td>
<td>BSO in PC12 Cells</td>
<td>inhibitory</td>
<td>Parkinson's Disease</td>
<td>Jha et al. 2002</td>
</tr>
<tr>
<td>α-ketoglutarate dehydrogenase</td>
<td>H₂O₂ in HEK293 cells</td>
<td>inhibitory</td>
<td>Alzheimer's Disease</td>
<td>Shi et al. 2008</td>
</tr>
</tbody>
</table>
Scheme 1-5: The NFκB Signaling Pathway is Regulated by Both Grx and Trx

NFκB activation occurs with phosphorylation of IκB which allows for the release and translocation of NFκB (shown as the two subunits, p50 and p65) to the nucleus. Inside the nucleus, NFκB binds DNA and activates transcription. This pathway has been previously reported to be regulated at numerous steps by both Grx and Trx as described in the text and illustrated here.
The other cellular pathway shown to be regulated by both Trx and Grx is the ASK1 pathway, a MAPKKK. MAPKs are a family of Serine-Threonine kinases that relay a signal the cytoplasm or cell surface to the nucleus. The three main families of MAPKs are ERKS (extracellular signal regulating protein kinases), p38, and JNK. MAPKs typically phosphorylate Thr-x-Tyr within conserved motifs (S. J. Harper, N. Wilkie 2003). These two families of MAPKs transduce different signals, and a key focus of this thesis (Chapter 3) is on the proapoptotic MAPKs, specifically JNK (cJun N terminal Kinase) and p38.

JNK isoforms are differentially expressed. JNK1 and JNK2 are widely expressed in all tissues. JNK3 exists in the brain and testis. Each of these subclasses of JNK has multiple isoforms (S. J. Harper, N. Wilkie 2003). JNK is activated by upstream kinases, M KK4 and M KK7 (Scheme 1-6, p 37), under a variety of stresses including UV light, hydrogen peroxide, heat shock, and growth factor withdrawal (S. J. Harper, N. Wilkie 2003). Importantly, JNK has been shown to be activated in a variety of PD models including 6-OHDA (M. Ouyang, X. Shen 2006), MPTP (D. S. Cassarino et al. 2000; S. Hunot et al. 2004; S. Karunakaran et al. 2007a), and dopamine treatment (D. Daily et al. 2001). A knockout mouse of JNK3 has been studied and shown to protect from a variety of stresses including oxygen deprivation, glucose deprivation, Aβ toxicity, NGF deprivation, and hypoxia/ischemia conditions (reviewed in (S. J. Harper, N. Wilkie 2003)). JNK phosphorylates several downstream transcription factors and regulates transcription. A few downstream targets are cJun, MADD (MAPK Activating Death Domain), p53, and ATF 2 (Activating Transcription Factor) (S. J. Harper, N. Wilkie 2003).
Recently, a small molecule inhibitor of the upstream activating molecule, MLK (Mixed Lineage Kinase), was tested in a clinical trial in PD patients. Unfortunately, this molecule (CEP1347) does not display therapeutic benefit (The Parkinson Study Group PRECEPT Investigators 2007). A number of reasons may account for the lack of therapeutic benefit including ineffective amounts of drug in the Substantia nigra or an ineffective target, i.e. MLK may not be the major MAPKKK activating JNK. As discussed in Chapter 3, ASK1 (described below) is the major upstream kinase activating JNK in a L-DOPA treated model, diminishing the likelihood that MLK is an ideal drug target for L-DOPA treated PD.

p38 also consists of differentially expressed isoforms. α and β isoforms are found in the brain, the γ isoform is found in skeletal muscle, and the δ isoform is found in the lung and kidney. p38 is activated by upstream MKK3 and 6 (Scheme 1-6, p 37) in response to a variety of conditions including treatments with 6-OHDA (Y. Ikeda et al. 2008; M. Gomez-Lazaro et al. 2008; Y. P. Hwang, H. G. Jeong 2008) or MPTP (S. Karunakaran et al. 2007a), oxidative stress, and ER stress (E. K. Kim, E. J. Choi 2010). Importantly, phosphorylated p38 indicative of apoptotic activation has been found in neurons of the Substantia nigra in post mortem patients with PD (I. Ferrer et al. 2001). Downstream, p38 activates a number of transcription factors including MAPKAPK 2,3,5, Elk-1 (Eph-like kinase), ATF 2, and CHOP (CCAAT/enhancer binding Homologous Protein). Knockout of p38α was embryonic lethal (M. Allen et al. 2000), prohibiting studies of neurodegeneration with this mouse model.

Both p38 and JNK require activation by upstream MAPKK and MAPKKK as shown in Scheme 1-6 (p 37). A major activating kinase of p38 and JNK is ASK1.
Coincidentally, this upstream MAPKKK is activated under conditions of oxidative stress, sending downstream signals to increase apoptosis.

1.15 Apoptosis Signaling Kinase 1 (ASK1) is Activated in Models of PD

ASK1 is a mitogen activated protein kinase kinase kinase (MAPKKK), whose activation in various contexts results in phosphorylation of alternative downstream substrates such as p38-MAPK or JNK, both associated with increased apoptosis (S. J. Harper, N. Wilkie 2003; I. G. Onyango et al. 2005; J. Pan et al. 2007; M. Miloso et al. 2008; W. Yang et al. 2009).

ASK1 is a central mediator of apoptosis. Thus, activation of this MAPKKK (mitogen activated protein kinase kinase kinase) results in phosphorylation of downstream substrates such as p38-MAPK or c-Jun N terminal kinase (JNK), both implicated in propagation of apoptosis (S. J. Harper, N. Wilkie 2003; I. G. Onyango et al. 2005; J. Pan et al. 2007; M. Miloso et al. 2008; W. Yang et al. 2009). A wide variety of signaling molecules activate this pathway, including growth factors, UV radiation, cytokines, and oxidative stress (K. Takeda et al. 2008) (Scheme 1-6, p 37). In addition, the activity of ASK1 is reported to be modulated by a variety of negative and positive effector proteins, forming the so-called signalosome. Many of these ASK1 modulators are sensitive to oxidative stress, including Grx1 and Trx1 themselves (A. Matsuzawa, H. Ichijo 2008).

As with most kinases, ASK1 activity is regulated by its pattern of phosphorylation. Phosphorylation at Ser83 and Ser 967 is an inhibitory modification (A. H. Kim et al. 2001; E. H. Goldman et al. 2004). Phosphorylation of Tyr718 targets ASK1
for degradation through its association with SOCS1 (Y. He et al. 2006). Phosphorylation of Thr 813, Thr838, and Thr842 are activating modifications leading to downstream phosphorylation of MAPKK (K. Tobiume et al. 2002). ASK1 preferentially phosphorylates serine and threonine residues on its target proteins. These proteins include mitogen activated protein kinase kinases (MAPKK) which in turn phosphorylate their downstream MAPK targets.

ASK1 is typically considered a pro-apoptotic protein; however, it may also activate the Erk pathway which is a pro-survival pathway (J. Matsukawa et al. 2004). ASK1 has been shown to be required for prolonged activation of p38 and JNK. ASK1 knockout mice show resistance to LPS-induced septic shock (Y. Sekine et al. 2006). Furthermore, ASK1 deficient cells show resistance to H₂O₂ treatment, and primary neurons from ASK1 knockout mice show resistance to Aβ toxicity (K. Takeda et al. 2008).

ASK1 activation has been reported in multiple PD models. For example, 6-OHDA treated rats displayed a loss of nigral striatal neurons due to activation of the ASK1-JNK pathway (J. Pan et al. 2007). In SHSY5Y cells, 6-OHDA leads to phosphorylation of downstream kinases, JNK and p38, and ultimately to cell death. Knockdown of ASK1 prevented apoptosis (M. Ouyang, X. Shen 2006). Furthermore, a MPTP mouse model of PD showed ASK1 activation leading to loss of DA neurons (S. Karunakaran et al. 2007a). Additional studies showed treatment of male mice with MPTP led to ASK1 activation. However, this activation of ASK1 was not seen in female mice, where the authors hypothesize protection due to the abundance of TR, GR, Grx and Trx in the female brain (U. Saeed et al. 2009).
In addition, paraquat treatment and endoplasmic reticulum stress lead to ASK1 activation concomitant with apoptosis in both cells (SHSY5Y) and mice (S. Karunakaran et al. 2007a; W. Yang et al. 2009; U. Saeed et al. 2009; K. Homma et al. 2009). Recently, induction of Trx by the transcription factor Nrf2 led to protection from paraquat through diminution of phosphorylation and activation of ASK1 (M. Niso-Santano et al. 2010).

Indeed, MAPKs play important roles in normal physiology as well as in pathological responses to adverse stimuli. Reported stimuli that activated MAPKs in the context of neurodegenerative diseases include dimerized amyloid precursor protein (APP), α-synuclein, 6-OHDA, MPTP, ROS, LPS, ER stress, and calcium influx (reviewed in (E. K. Kim, E. J. Choi 2010)).

1.16 ASK1, Redox Regulation by Trx and Grx

Apoptosis signaling kinase 1 (ASK1) has been shown to be activated in response to a number of stimuli (see Scheme 1-6, p 37) including oxidative stress. Moreover, thiol homeostatic enzymes Grx and Trx have been reported to directly bind to and inhibit activation of ASK1. Grx has been shown to bind to the C-terminus of ASK1 (J. J. Song, Y. J. Lee 2003) and in an analogous fashion, Trx binds to the N terminus of ASK1 (M. Saitoh et al. 1998). Release of either Grx or Trx from ASK1 under oxidative conditions is sufficient to activate ASK1 and initiate downstream signaling through JNK and promote apoptosis (M. Saitoh et al. 1998; J. J. Song et al. 2002). In MCF-7 breast cancer cells dissociation of Grx from ASK1 was observed following oxidative stress via glucose deprivation (J. J. Song, Y. J. Lee 2003). This dissociation was inhibited with overexpression of catalase or addition of N-acetyl cysteine implicating ASK1 activation.
Scheme 1-6: Activation of ASK1 Occurs in Response to a Variety of Stimuli Including Oxidative Stress

ASK1 is activated under a number of different stresses arising from various components of the cell including the ER. Once activated, ASK1 phosphorylates downstream MAPKKs which leads to phosphorylation of MAPKs, p38 and JNK. This cell signaling cascade leads to cell death.
as a response to oxidative challenges. Depletion of cellular GSH by treatment with buthionine sulfoximine (BSO) is reported to inhibit dissociation of Grx from ASK1 during glucose deprivation. Despite the oxidizing environment, the ASK1-Grx1 complex remains intact, prohibiting downstream JNK activation. The authors offer the rationalization that loss of GSH inhibits formation of the intramolecular disulfide in the active site of Grx1 (J. J. Song et al. 2002). This explanation is problematic since depletion of GSH should favor accumulation of the intramolecular disulfide form of Grx, not the opposite, and this should promote dissociation from ASK1. Another, more likely mechanism involves direct modification of the ASK1 protein, as seen previously with NEM activation of ASK1 via direct modification of Cys residues on ASK1 (J. V. Cross, D. J. Templeton 2004).

Several studies have suggested a role for Trx in sequestering the apoptotic factor, ASK1, in an inactive conformation within the cytosol by binding to its N-terminus (M. Saitoh et al. 1998). Trx requires its active “CXXC-motif” to bind to ASK1, and only upon oxidation to its intramolecular disulfide form is the Trx released, enabling ASK1 to activate proteins that initiate apoptotic signaling, including c-Jun-N-terminal kinase (JNK) and members of the p38 MAPK pathway (G. Powis, W. R. Montfort 2001). As discussed earlier, Grx can bind to the C-terminus of ASK1, apparently contributing also to inhibition of ASK1 in the cytosol (J. J. Song, Y. J. Lee 2003). Binding to opposite ends of ASK1 suggests that Trx and Grx may regulate ASK1 independently under different conditions. Unfortunately, the majority of ASK1 protein interaction studies have been performed under conditions of overexpression of usually both ASK1 and either
Grx or Trx. This provides evidence that these proteins can interact, however it is still unknown whether such interactions occur under normal physiological conditions.

1.17 Summary, Rationale, and Aims of the Current Studies.

Parkinson’s disease is associated with increased oxidative stress coupled with a decreased antioxidant capacity. L-DOPA is the main therapy for PD; however, L-DOPA can also increase oxidative stress within cells potentially exacerbating the disease state. Pro-oxidant therapy in an already oxidative environment primes the cell for modification of thiols on proteins potentially altering their native function. Modifications can occur leading to loss or gain of function as well as targeting the proteins for degradation. The main modification of protein thiols, glutathionylation, may play a pivotal role in the initiation of PD and/or may be a consequence of disease progression. Furthermore, protein glutathionylation may occur in the early stages of the disease as a protective mechanism combating oxidative challenges within the brain but may be unable to be reversed. To date, no studies have addressed mechanism(s) by which L-DOPA influences thiol homeostasis nor mechanism(s) by which L-DOPA induces apoptosis in cell culture models.

We hypothesized that L-DOPA treatment of dopaminergic neurons leads to increased oxidative stress and thus increased oxidative modifications on proteins altering their normal functions. Normally, oxidative modifications are reduced by thiol homeostatic enzymes; however, perturbations in these regulatory enzymes through loss of function result in persistence of oxidized proteins. Overt oxidative challenge without sufficient enzymatic reduction leads to increased apoptosis. We chose to test this
hypothesis in SHSY5Y cells (acquired from the American Type Culture Collection (ATCC)). These cells are isolated from bone marrow neuroblastoma and were derived 3 times to obtain a homogenous sample of dopaminergic-like neuronal cells to facilitate a straightforward approach to study the molecular mechanisms of L-DOPA-induced cell injury. This cell line displays typical dopaminergic characteristics, namely expression of tyrosine hydroxylase, dopamine transporter, vesicular monoamine transporter 2, dopamine receptor 2, and monoamine oxidase A and B; and these cells also contain dopamine and dopamine metabolites (A. Klegeris, P. L. McGeer 2000; H. Legros et al. 2004). Throughout this thesis, for simplicity, these neuroblastoma cells are referred to as SHSY5Y neurons. The aims of this study were to (1) determine thiol homeostatic enzyme alteration by L-DOPA in SHSY5Y neurons, (2) elucidate the impact of any changes on cell survival, and (3) discover a cell death pathway regulated by these thiol homeostatic enzyme systems responsible for L-DOPA induced cell death.

In this study, we found L-DOPA induces apoptosis in a dose-dependent manner. Negative effects of L-DOPA on cell fate are potentiated 3-4 fold by depletion of cellular GSH (Chapter 2). Apoptosis induced by L-DOPA treatment under diminished GSH conditions occurred at drug concentrations in the range of those that occur with doses prescribed to patients (G. Walkinshaw, C. M. Waters 1995). L-DOPA alters the thiol homeostatic enzymes, Grx, Trx, and TR by different mechanisms. Grx appears to be inactivated in SHSY5Y neurons treated with L-DOPA by dopaquinone adduction to the active site Cys 22. These results were corroborated by studies of concentration- and time-dependent loss of enzymatic activity of Grx1 treated with oxidized L-DOPA, and mass spectral identification of the nature of the modification with the isolated Grx1
protein. Trx and TR are diminished in content (and equally in activity due to loss of enzyme) under L-DOPA treatment. Loss of either Grx or the Trx enzyme system leads to increased apoptosis (Chapter 2). Having observed loss of both enzyme systems, we focused on cell fate pathways regulated by both Grx and Trx, namely NFκB and ASK1. We found no changes (increased or decreased) in NFκB activity in response to L-DOPA. However, ASK1 was activated with L-DOPA treatment revealed by phosphorylation of p38 and JNK occurring in a dose-dependent manner. Inhibition of either p38 or JNK protected SHSY5Y neurons from L-DOPA induced apoptosis, and dual inhibition of p38 and JNK implicated a synergistic relationship since a combination of low, ineffective doses of the two inhibitors did afford protection from L-DOPA-induced apoptosis. Knockdown of ASK1 nearly abolished L-DOPA induced apoptosis, implicating ASK1 as the major cell death pathway activated by L-DOPA in SHSY5Y neurons (Chapter 3).

Taken together, these data elucidate the primary cell death mechanism involved in L-DOPA induced apoptosis. Inhibition of the Trx system and inactivation of Grx results in alteration in protein thiol status disabling the reduction of oxidized proteins. Many pathways could be affected by loss of either of these enzymes; however, we found ASK1 is the major pathway affected by loss of these enzymes, resulting in its activation and induction of apoptosis. As ASK1 is the major pathway involved in L-DOPA induced apoptosis, future studies addressing its ability to be inhibited therapeutically could diminish adverse effects from L-DOPA treatment.
Chapter 2: Levodopa Deactivates Enzymes that Regulate Thiol-Disulfide Homeostasis and Promotes Neuronal Cell Death: Implications for Therapy of Parkinson’s Disease

Part of the work presented in Chapter 2 was published in

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2.1 Abstract:

Parkinson’s disease (PD) is characterized by dopaminergic neuronal loss, and is attributed to oxidative stress, diminished glutathione (GSH), mitochondrial dysfunction, and protein aggregation. Treatment of PD involves chronic administration of Levodopa (L-DOPA) which is a pro-oxidant and may disrupt sulfhydryl homeostasis. The goal of current studies is to elucidate the effects of L-DOPA on thiol homeostasis in a model akin to PD, *i.e.* immortalized dopaminergic neurons (SHSY5Y cells) with diminished GSH content. These neurons exhibit hypersensitivity to L-DOPA-induced cell death, which is attributable to concomitant inhibition of the intracellular thiol disulfide oxidoreductase enzymes. Glutaredoxin (Grx) was deactivated in a dose-dependent fashion; but its cellular content was unaffected. Glutathione disulfide (GSSG) reductase (GR) activity was not altered. Selective knockdown of Grx resulted in increased apoptosis, documenting the role of the Grx system in neuronal survival. L-DOPA treatments also led to decreased activities of thioredoxin (Trx) and thioredoxin reductase (TR), concomitant with diminution of their cellular contents. Selective chemical inhibition of TR activity led to increased apoptosis, documenting the Trx system’s contribution to
neuronal viability. To investigate the mechanism of inhibition at the molecular level the isolated enzymes were each treated with oxidized L-DOPA. GR, Trx, and TR activities were little affected. However, Grx was inactivated in a time- and concentration-dependent fashion indicative of irreversible adduction of dopaquinone to its nucleophilic active site Cys 22, consistent with intracellular loss of Grx activity but not content after L-DOPA treatment. Overall L-DOPA is shown to impair the collaborative contributions of the Grx and Trx systems to neuron survival.
2.2 Introduction:

Parkinson’s disease (PD), the second most common neurodegenerative disease, is characterized by bradykinesia, resting tremor, rigidity, and postural instability. PD affects mostly catecholaminergic neurons in the substantia nigra of the brain (P. F. Riederer 2004), and it is linked to both genetic and environmental factors, involving oxidative stress, mitochondrial dysfunction, and protein aggregation (K. A. Maguire-Zeiss et al. 2005). Many reports have attributed the pathogenesis of PD to the oxidative stress concomitant with increased dopamine turnover, deficient glutathione content, and increased iron in the substantia nigra (C. W. Olanow, W. G. Tatton 1999).

The primary agent for current therapy of PD is L-DOPA, a precursor of dopamine, serving to restore dopaminergic signals from catecholaminergic neurons. Typically L-DOPA is used at increasing doses as the disease progresses to provide a therapeutic effect. Furthermore, L-DOPA treatment of rodents is reported to increase lipid peroxidation, characteristic of oxidative stress (C. W. Olanow, W. G. Tatton 1999). Exposing cultured pheochromocytoma cells (PC12 cells) to increasing concentrations of L-DOPA has led to cell detachment resulting in cell death (G. Walkinshaw, C. M. Waters 1995) and Figure 2-1 (p 45). These similar (both reported in (G. Walkinshaw, C. M. Waters 1995) and in our hands) outcomes are attributed to metabolism of L-DOPA creating reactive oxygen species (ROS), superoxide and hydrogen peroxide which could promote both reversible (disulfide and sulfenic acid) and irreversible (sulfinic and sulfonic acids) modifications of protein Cys residues (Scheme 1-2, p 9). Furthermore, highly reactive quinone species can be formed from L-DOPA and its products, dopamine
**Figure 2-1**: L-DOPA Induces Cell Death in PC12 Cells

Bar graph of percent apoptotic PC12 cells following treatment. PC12 cells were treated with L-DOPA (gray) or etoposide (black) and analyzed 24 hr (L-DOPA) or 48 hr (etoposide, 10 μM) after treatment for chromatin condensation as observed by Hoechst 33342 dye (see Methods). Etoposide was used as a positive control as this drug facilitates chromatin condensation. Samples were counted in triplicate in a blinded fashion. Samples represent n=2 (biological replicates).
and DOPAC, which may react with exposed cysteine (Cys) residues on proteins (Scheme 1-1, p 6).

Since Walkinshaw and Waters previously showed L-DOPA induced cell death in PC12 cells and in our hands these cells were not sensitive to L-DOPA within a physiological range as determined by peak plasma levels (2010), we chose to continue experiments in SHSY5Y neurons.

Studies performed with isolated L-DOPA show spontaneous and non-enzymatic oxidation to ROS and reactive quinones (M. Asanuma et al. 2004). Such reactive quinone species can irreversibly conjugate to Cys residues on proteins, forming S-cysteinyldopamine adducts, thereby altering protein function (K. A. Maguire-Zeiss et al. 2005). Moreover, there is evidence that this may occur in PD patients. Thus, post mortem human brain tissue samples from L-DOPA treated versus control showed a significant increase in quinone adducts with the highest quantities found within the substantia nigra (J. P. Spencer et al. 1996).

A recent review of clinical trials focused on potential L-DOPA toxicity in patients portrayed an ambiguous picture and suggested further clinical and basic science studies are necessary to advance understanding (J. E. Ahlskog 2007). The ELLDOPA (early versus late levodopa) clinical trial suggested that L-DOPA does not accelerate progression of PD based on Unified Parkinson’s Disease Rating Scale (UPDRS) scores. Neuroimaging studies examining PD brains before and after L-DOPA treatment from patients in the same ELLDOPA study indicate increased decline with L-DOPA treatment. Elevated $\beta$-CIT (methyl-3$\beta$-(4-iodophenyl)-tropane-2 $\beta$-carboxylate) uptake measuring straital dopamine transporter uptake was observed in the L-DOPA group compared to the
placebo group. Thus, further investigation is warranted to determine the risks and benefits of L-DOPA in the treatment of PD.

In this thesis study, biochemical and molecular mechanisms underlying the potential consequences of L-DOPA therapy were explored using a PD cell culture model, SHSY5Y immortalized dopaminergic neurons. These cells exhibit dopaminergic characteristics including expression of tyrosine hydroxylase, and production of dopamine and its metabolites (A. Klegeris, P. L. McGeer 2000; H. Legros et al. 2004). Since L-DOPA metabolism can promote thiol oxidation (M. Asanuma et al. 2004) and thiol homeostasis is important for cell survival, we investigated L-DOPA-induced apoptosis and focused on alterations in thiol homeostatic enzymes within SHSY5Y neurons.

Here we report that L-DOPA treatment of SHSY5Y neurons leads to apoptotic cell death. Concomitantly, Grx is deactivated without change in its cellular content. Furthermore, there are partial losses of TR and Trx activities upon L-DOPA treatment, with corresponding losses in their contents, suggesting a different mechanism of deactivation. Biochemical studies of the isolated enzymes showed only Grx, and not GR, Trx, or TR, is inactivated substantially by exposure to oxidized L-DOPA leading to a specific active-site adduct. Selective knockdown of Grx or selective chemical inhibition of TR each led to increased apoptosis. These model studies suggest that disruption of thiol homeostasis through deactivation of key enzymes may contribute to overall loss of dopaminergic neurons in PD patients during therapy with L-DOPA.
2.3 Results:

Diminution of [GSH] in SHSY5Y cells leads to increased sensitivity to L-DOPA: In Parkinson’s disease brains the Substantia nigra has a decreased antioxidant capacity mainly through diminution of GSH levels (T. L. Perry et al. 1982; P. Jenner et al. 1992; J. Sian et al. 1994). To examine whether diminution of GSH leads to increased sensitivity to the oxidative insult associated with L-DOPA treatment, we decreased the GSH content by treatment of the cells with buthionine sulfoximine (BSO, 0.1mM). Treatment with BSO provided approximately 80% GSH depletion at 24 hr in immortalized dopaminergic neurons (SHSY5Y cells) (Control: 0.68 mM ± 0.06, BSO treated: 0.13 mM ± 0.01, Figure 2-2, p 49). A similar loss of GSH content in the substantia nigra is seen for patients with PD (T. L. Perry et al. 1982).

To assess cell sensitivity to L-DOPA, several assays of cell death were performed. Treatment of SHSY5Y cells for 24 hr with L-DOPA led to a dose-dependent decrease in cell viability according to loss of MTT reactivity, and the dose-response curve was shifted to the left in cells that were co-treated with 0.1 mM BSO (Figure 2-3, p 50). The dose-response relationship for L-DOPA-dependent increase in cell death (trypan blue analysis) was also shifted to lower concentrations in GSH-deficient cells (Figure 2-4, p 51). Apoptosis (according to chromatin condensation) increased with increasing L-DOPA, occurring at lower concentrations with BSO co-treatment. The majority of cell death is attributed to apoptosis since quantification of cells positive for chromatin condensation (Hoechst staining) corresponded closely to the extent of cell death assessed with trypan blue (Figure 2-5, p 52). Sensitivity to L-DOPA cytotoxicity was increased
**Figure 2-2:** Buthionine Sulfoximine Depletes GSH in a dose dependent fashion

![Bar graph illustrating GSH content in SHSY5Y cells. Buthionine sulfoximine (BSO) was added to diminish cellular GSH levels. BSO treatment depleted GSH in a dose dependent fashion. 0.1mM BSO was chosen to mimic PD conditions yielding 80% diminution of GSH at 24 hours. It appears that the maximal effect of the drug is seen at 0.5mM BSO treatment potentially, due to the time frame of treatment as BSO simply inhibits the rate limiting enzyme in GSH anabolism. n=3 (biological replicates).]
Figure 2-3: L-DOPA reduces Cell Viability in SHSY5Y Cells as Measured by MTT Reduction

Bar graph displaying relative cell viability as measured by the MTT assay (see Methods) when treated with L-DOPA for 24 hr. Solid bars represent cells with replete GSH. Open bars indicate cells treated with 0.1mM BSO. All samples were normalized to the untreated control. BSO treatment led to a ~3-fold increase in sensitivity to L-DOPA. * represent significant difference compared to control, p<0.01 (n = 3 (biological replicates)).
**Figure 2-4:** L-DOPA causes Cell Death in a Dose Dependent Fashion in SHSY5Y cells. GSH depletion sensitizes cells to L-DOPA.

Dose response curves for L-DOPA dependent cell death as measured by trypan blue exclusion. SHSY5Y cells were treated with L-DOPA for 24 hr and trypan blue was used to measure cell death, as described in Methods. Treatment in the presence of BSO led to ~3-fold sensitization of SHSY5Y cells to L-DOPA induced cell death. ■ symbols represent BSO ± L-DOPA treated cells, giving an EC50 ~ 40μM; ♦ represent L-DOPA only treated samples, with an EC50 of ~ 100μM. Curves were fitted to the following equation:

\[
\% \text{ dead} = \text{Effect}_{\text{max}} \times [\text{L-DOPA}]^n / (EC_{50})^n \pm [\text{L-DOPA}]^n,
\]

where n is equivalent to the Hill coefficient.
**Figure 2-5:** Diminution of [GSH] in SHSY5Y Cells leads to Increased Sensitivity to L-DOPA Induced Apoptosis

Dose-response curves for L-DOPA dependent apoptosis as measured by chromatin condensation (Hoechst staining). SHSY5Y cells were treated with L-DOPA for 24 hr and apoptosis was measured using Hoechst 33342 dye (see Methods). Samples were counted in triplicate in a blinded fashion. ■ symbols represent BSO ± L-DOPA treated cells; ♦ represent L-DOPA only treated samples. The curves were fit to the % apoptosis vs. [L-DOPA] data according to the following equation, minimizing the least squares deviation, and normalized to the control, setting no treatment to zero:

\[
\% \text{ apoptosis} = \text{Effect}_{\text{max}} \cdot [\text{L-DOPA}]^n / (\text{EC}_{50})^n \pm [\text{L-DOPA}]^n, \text{ where } n \text{ is equivalent to the Hill coefficient.}
\]

The EC\textsubscript{50} values for L-DOPA induced apoptosis calculated from the fitted curves are 49 \(\mu\)M (plus BSO) and 127 \(\mu\)M (minus BSO).
**Figure 2-6: Caspase 3 Activity Is Increased By Low L-DOPA Concentrations in SHSY5Y Cells with Diminished GSH Content**

Bar graph representing caspase 3 activity. SHSY5Y cells were treated with L-DOPA and 0.1mM BSO simultaneously and caspase 3 activity was measured fluorometrically 8 hr after treatment (see Methods). Activity initially increases and then decreases at higher drug concentrations.
approximately three-fold, according to all assays, when GSH was depleted by co-treatment with BSO.

A second measure of apoptosis was performed through analysis of caspase 3 activation under L-DOPA treated conditions. Initial studies were performed on cells treated with BSO concomitantly with L-DOPA. As shown in Figure 2-6 (p 53), caspase 3 activation increases upon L-DOPA treatment, however, at higher concentrations of L-DOPA, caspase 3 activity is diminished. This biphasic response could occur with inhibition of procaspase 3 through oxidative modification as described by others, where upon Fas treatment of Jurkat cells resulted in S-nitrosylation of caspase 3 and inhibition of its activity (J. B. Mannick et al. 1999). Due to the complexity of caspase 3 regulation in a pro-oxidant environment, this method was abandoned. Having documented L-DOPA dose-dependent cell death with SHSY5Y neurons, we wanted to examine potential mechanisms involving perturbation of sulfhydryl homeostasis.

Metabolism of L-DOPA can generate ROS and reactive quinones (Scheme 1-2, p 9); all of which can lead to protein-SH modifications. The greatest impact of such reactivity would be realized if the homeostatic enzymes themselves (Scheme 1-1, p 6) were modified by L-DOPA by-products. The two principal enzyme systems that mediate sulfhydryl homeostasis within cells are thioredoxin (Trx) and glutaredoxin (Grx), which catalyze the reduction of oxidized cysteine moieties (D. M. Ziegler 1985; J. J. Mieyal et al. 1995; E. A. Sabens, J. J. Mieyal 2009) (see Scheme 1-3, p 10). Thioredoxin effectively reduces intramolecular and intermolecular disulfides as well as sulfenic acids (C. Jacob et al. 2006). Glutaredoxin specifically reduces protein-glutathione mixed disulfides (Protein-SSG) (M. M. Gallogly et al. 2009). The different specificities suggest
Figure 2-7: L-DOPA Treatment Inhibits Grx Activity with No Change in Content
(A) Typical western blot showing Grx content in lysates of SHSY5Y cells, relative to actin (loading control). (B) Bar Graph displaying densitometric quantification of western blots for Grx content in SHSY5Y cells under various conditions. Solid bars represent cells with replete GSH. Open bars indicate cells treated with 0.1mM BSO. No statistically significant change in Grx content is evident, n=3 (biological replicates), p>0.5 for most comparisons. The content value for 100μM L-DOPA treatment tends toward an anomalous, but statistically insignificant increase in Grx content (n=8 (biological replicates), p=0.17). (C) Bar Graph displaying Grx activity assayed according to release of radiolabel ([3H] GSSG) from the prototypical substrate, [3H] BSA-SSG. Solid bars represent cells with replete GSH. Open bars indicate cells treated with 0.1mM BSO. Grx activity for control is 0.85 ± 0.21 nmol/min/mg protein; Grx activity for BSO treated control is 0.70 ± 0.19 nmol/min/mg protein. BSO provided ~2 fold increase in loss of Grx activity (by comparing the L-DOPA concentrations used to the loss of Grx activity compared to control). * represent significant difference compared to control, p<0.01 (n≥3).
**Figure 2-8:** GSSG Reductase (GR) is Not Inhibited by L-DOPA Treatment of SHSY5Y Cells

Bar graph of GR Activity. GR activity was measured spectrophotometrically as described under Methods for SHSY5Y cells treated with L-DOPA, either in the absence of BSO (Gray bars), or in the presence of 0.1mM BSO (decreased GSH, white bars). GR activity with replete GSH (gray bars) was not significantly changed. GR activity for the control is 2.2 ± 0.3 U/mg protein. GR activity for BSO treatment (white bars) was not significantly changed where the BSO control is 1.81 ± 0.19 U/mg total protein.
Figure 2-9: L-DOPA Treatment of SHSY5Y cells Results in Loss of Content and Activity of TR and Trx
(A) Typical western blot showing TR1 and Trx immunoreactivity relative to actin (loading control). (B) Bar graph of relative contents. **Left panel – replete GSH** densitometric quantification of content of TR1 (white bars) and Trx (black bars). **Right panel – diminished GSH:** densitometric quantification of content of TR1 (white bars) and Trx (black bars). Quantification was performed using Quantity One software. All samples were normalized to actin, the loading control. * indicates p<0.05, n=3 (biological replicates). (C) **Left panel – replete GSH:** Trx catalytic activity (black bars) showed significant L-DOPA concentration-dependent decreases, * p<0.05, (n=3 biological replicates). Trx activity measured for the control is 1.76 ± 0.04 U/mg protein. TR activity (white bars) showed significant L-DOPA concentration-dependent decreases, * p<0.05, (n=3 biological replicates). TR activity measured for the control is 1.98 ± 0.32 U/mg protein. **Right panel – diminished GSH:** Trx activity (black bars) showed small but significant L-DOPA concentration-dependent decreases, * p<0.05, (n=3 biological replicates). Trx activity for the control is 1.91 ± 0.26U/mg protein. Decreases in TR activity (white bars) indicated a 5-fold increased sensitivity to L-DOPA when GSH was diminished. TR activity for the control is 1.92 ± 0.07 U/mg protein.
that the Trx and Grx systems act synergistically to maintain cellular sulfhydryl homeostasis. Therefore, we investigated how L-DOPA treatment affected the activities of these enzymes.

*Thiol Disulfide Oxidoreductase Enzymes are Affected Differentially by L-DOPA Treatment:* In other contexts, Grx has been reported to be altered under oxidative stress conditions, resulting in changes in activity and/or content (reviewed in (J. J. Mieyal et al. 2008)). Therefore, we examined how L-DOPA affected both Grx content and activity. L-DOPA, even at the highest concentration used, did not decrease Grx content (*Figure 2-7 A, 2-7 B*, p 55). In contrast, Grx activity was diminished in a dose-dependent fashion with increasing concentrations of L-DOPA (*Figure 2-7 C*, p 55). Co-treatment with BSO led to more than a two-fold increased sensitivity of Grx to inhibition by L-DOPA treatment as shown in *Figure 2-7 C*, p 55. In contrast to Grx, the coupling enzyme for the Grx system, GR, was essentially insensitive to L-DOPA. *Figure 2-8*, p 57, shows the enzymatic activity of GR is essentially unaffected by concentrations of L-DOPA up to 100μM, and <20% inhibited at 250μM L-DOPA. With the Trx system, the activities of TR and Trx were decreased in an L-DOPA-dependent manner (*Figure 2-9 A*, p 58). Unlike Grx, in cells with replete GSH content, the loss of TR and Trx activities corresponded to proportional losses of content of the respective proteins as documented by western blot analysis (*Figure 2-9 B and 2-9 C*, p 58). Furthermore, Trx did not display increased sensitivity with BSO treatment, showing little inhibition (< 20%). Unlike Trx and similar to Grx, TR was more sensitive (~3-4 fold) to inhibition from L-DOPA treatment in the GSH-depleted cells (BSO-treated), and in this case TR1 content
Figure 2-10: GSH Content Increases with L-DOPA Treatment in SHSY5Y Cells with Replete GSH. BSO treatment Inhibits Increase in GSH Levels in Response to L-DOPA.

(A) Bar graph showing GSH content in SHSY5Y cells with replete GSH. n=3 (biological replicates)  
(B) Bar graph showing GSH content in SHSY5Y cells with diminished GSH (see methods). n=3 (biological replicates), p values are indicated on the figure showing a significant difference between BSO/L-DOPA treatment and BSO treatment alone. In both (A) and (B) initial GSH conditions (i.e. untreated or BSO alone) were set to 100% and the effect of L-DOPA on GSH levels was normalized to these untreated, control levels of GSH.
Figure 2-11: Grx is the only Thiol Disulfide Oxidoreductase Inactivated by Oxidized L-DOPA.
(A) Isolated Grx was incubated with oxidized L-DOPA (preparation described under the Methods section), and aliquots of the reaction mixture were withdrawn every 10 minutes and assayed for enzyme activity as described in Methods. Grx activity was decreased in a time and dose-dependent fashion. (B) Replot of Grx activity data from (A) on a semi-log scale showing straight lines with increasing negative slopes indicative of pseudo-first order enzyme inactivation. (C) Each isolated enzyme was incubated in the absence or presence of oxidized L-DOPA, assayed every 20 minutes for the respective enzyme activities as described under Methods. GR, Trx and TR activities were unaffected, even when tested in separate experiments where the aliquots were withdrawn at a 60 minute interval. In contrast, Grx was inactivated in an oxidized L-DOPA-dependent manner; * represents significant differences relative to control, p<0.015 (n=3 biological replicates).
did not change. These findings suggest a shift in mechanism of inhibition of the Trx system when GSH is depleted (see Discussion).

Upon examining GSH content after L-DOPA treatment, it is noteworthy that GSH content in cells with replete GSH underwent a slight increase in GSH levels in response to L-DOPA treatment (Figure 2-10 A, p 61). This result is thought to be a cellular protective response to oxidative stress induced by drug treatment. However, cells treated with BSO, which inhibits GSH synthetase, did not display this increase in GSH protective response (Figure 2-10 B, p 61), as expected.

**Oxidized L-DOPA Grx Selectively Inactivates Grx In Vitro:** As described above, quinone adduction is a potential mechanism by which L-DOPA treatment may lead to inhibition of the thiol-disulfide oxidoreductases. Therefore, we studied time- and concentration-dependent inactivation of the purified enzymes by oxidized L-DOPA. Grx was inactivated in a dose-dependent fashion according to pseudo-first order kinetics (Figure 2-11 A & B, p 62). Addition of GSH to the pre-incubation mixture protected Grx, consistent with data from cells with replete GSH (Figure 2-7 C, p 55). Inactivation of Grx by quinone adduction would be expected to involve the active site Cys 22, which possess a unique thiol pKa of 3.5 (see below).

The other thiol disulfide oxidoreductases were tested with oxidized L-DOPA in an analogous fashion. GR, Trx, and TR showed very little sensitivity to oxidized L-DOPA (Figure 2-11 C, p 62). While most of the effects on enzyme activities in the cell studies are consistent with the observations with the isolated enzymes, the lack of adductive inactivation of the isolated TR contrasts remarkably with the loss of TR
activity without the loss of the TR protein in GSH-deficient cells treated with L-DOPA (see Discussion).

*Dopa-quinone adducts to Grx at the Active Site:* In order to determine the site(s) of covalent quinone modification resulting in Grx inactivation, purified Grx protein was treated with oxidized L-DOPA and then analyzed by mass spectrometry. After inactivation of the Grx, we observed an increase of 194 mass units in the deconvoluted mass spectrum (*Figure 2-12 A*, p 66). This is indicative of adduction of 1 dopaquinone to Grx. Upon trypsin digestion of the protein and fragmentation of the tryptic peptides using MS/MS, the adduction at the active site was confirmed. A mass shift of 194 m/z was detected at Cys 22 when the untreated protein (*Figure 2-12 B*, p 66) was compared to the inactivated protein (*Figure 2-12 C*, p 66). Modification at this site is consistent with inactivation because Cys 22 is required for the catalytic activity of Grx1. Despite the over abundance of oxidized L-DOPA, only Cys 22 was adducted by dopaquinone. Mass spectrometry was performed and analyzed by Anne Distler, Ph.D.

*Grx knockdown in SHSY5Y cells leads to Apoptosis:* Although a loss of Grx activity is apparent, other events may contribute to L-DOPA-dependent increases in apoptosis. Therefore, we tested whether selective loss of Grx activity would result in increased apoptosis by using siRNA directed against Grx1 production in SHSY5Y cells. The extent of knockdown of Grx1 was confirmed by western blot (*Figure 2-13 A*, p 67).
Figure 2-12: Quinone Adduction Occurs on the Active Site Cys 22 of Grx.

(A) Adduction of 194 Da was seen with the sample treated with oxidized L-DOPA (bottom) compared to the untreated sample (top). (B) MS/MS spectrum of untreated Grx. Fragmentation shows complete coverage of the active site. (C) MS/MS spectrum of L-DOPA treated Grx. Fragmentation shows adduction of 194 m/z to Cys 22 within the active site. All mass spectral data and analysis was contributed by Anne Distler, Ph.D. Nomenclature originally developed by Klaus Biemann was used to describe the fragmentation pattern. Y ions are depicted as the charge was maintained from the C terminus, and subsequently few b ions were detected.
Figure 2-13: Knockdown of Grx Increases Apoptosis in SHSY5Y Cells

(A) Typical western blot confirming Grx1 knockdown relative to controls and compared to actin (loading control). (B) Bar graph showing densitometric quantification of Grx1 knockdown. (C) Bar graph showing relative apoptosis for control SHSY5Y cells vs. cells in which Grx1 was knocked down (Grx1-siRNA). Apoptosis was measured in triplicate by chromatin condensation in a blinded fashion using Hoechst 33342 dye. * indicates p<0.01, n=3 independent biological samples.
We chose to knock down Grx1 by 50% to approximate the amount of residual active Grx observed with 100μM L-DOPA treatment of the cells with replete GSH (Figure 2-7 C, p 55). Knockdown of Grx1 by ~50% led to ~30% increase in apoptosis, as measured by nuclear chromatin condensation compared to cells transfected with scrambled siRNA or mock transfected cells. (Figure 2-13 B, p 67) A 30% increase in apoptosis accounts for most of the apoptosis observed with 100μM L-DOPA treatment under conditions of replete GSH. This result further implicates Grx as an important regulator of cell death within SHSY5Y cells. We note an increase in cell death with introduction of scrambled siRNA compared to mock treated cells (i.e., lipofectamine 2000 alone). We expect that this slight toxicity is seen as a side effect of introducing RNAi. According to the manufacturer’s protocol, cells exhibit hypersensitivity to being exposed to double stranded RNA, therefore the knockdown cells are directly compared to scrambled controls.

_Auranofin Induced Inhibition of TR leads to Apoptosis:_ To inhibit TR selectively, we used the well characterized chemical inhibitor, auranofin (A. G. Cox et al. 2008; V. Gandin et al. 2009) and titrated its concentration to inhibit TR to a similar extent as that seen with 100μM L-DOPA treatment of the cells with replete GSH. Treatment of SHSY5Y cells with auranofin led to increased apoptosis in a dose-dependent fashion (Figure 2-14 A, p 69), in parallel with increasing inhibition of TR (Figure 2-14 B, p 69). These results support the conclusion that TR activity also is important for cell survival in SHSY5Y cells.
Figure 2-14: Inhibition of TR increases Apoptosis in SHSY5Y Cells

(A) Bar graph showing auranofin concentration-dependent increase in apoptosis of SHSY5Y cells. Apoptosis was measured 24 hr after treatment according to chromatin condensation (Hoechst staining). * indicates p<0.01, n=3 (biological replicates); measurements done in triplicate. (B) Bar graph showing auranofin concentration-dependent inhibition of TR in SHSY5Y cells. Auranofin was titrated to give inhibition of TR equal to that seen with 100μM L-DOPA treatment in GSH replete cells. #indicates p<0.05, * indicates p<0.01, n=3 (biological replicates).
2.4 Discussion:

We discovered that L-DOPA treatment leads to loss of activity of the Grx and Trx systems in SHSY5Y neurons. Grx deactivation by L-DOPA was dose-dependent, and it was potentiated in GSH depleted cells occurring without change in Grx content (Figure 2-7, p 55). TR and Trx activities were diminished after L-DOPA treatment of the SHSY5Y neurons with replete GSH in proportion to loss of their respective contents (Figure 2-9, p 58). In cells with depleted GSH, however, TR was inhibited by L-DOPA, but its content was unchanged. The following considerations address the different modes of inactivation of the Grx and Trx systems. (see below).

In this study we investigated potential mechanisms by which L-DOPA treatment alters thiol homeostasis leading to cell death in immortalized SHSY5Y neurons. It is noteworthy that the range of concentrations of L-DOPA that are neurotoxic in GSH deficient SHSY5Y cells (this study) overlaps the range of concentrations provided in The Pharmacological Basis of Therapeutics (2010) in PD patients whose neurons are not only deficient in GSH but also are impaired in mitochondrial function. Maximal L-DOPA plasma concentrations in patients taking 150mg L-DOPA (in combination with Carbidopa, the amino acid decarboxylase inhibitor preventing peripheral metabolism of L-DOPA) ranged from 6-13μM. Patients can take as much as 250mg L-DOPA at one time resulting in approximately 20μM peak plasma concentration. However, cerebral spinal fluid samples taken by lumbar puncture from patients were approximately 10-fold less than levels seen in the plasma (P. Benetello et al. 1997). More study must be performed to understand the actual L-DOPA concentrations within neurons which are currently unknown. We realize there is a ten-fold difference or more between in the
concentration of L-DOPA in the CSF compared to what we are using in cell culture, and we also understand that our cell line, despite being dopaminergic, does not contain all characteristics of PD dopaminergic neurons. We have simulated one aspect of the PD cellular milieu by diminishing GSH content with BSO treatment; however, other pertinent characteristics that may also increase the sensitivity of L-DOPA were not simulated, including loss of complex I activity, increased iron content, and decreased antioxidant system including diminished ROS scavenging enzymes (see Chapter 1) (K. A. Maguire-Zeiss et al. 2005).

The Grx System: In previous studies, our research group learned that the cyclic disulfide-containing fungal toxin sporidesmon inactivates Grx by covalent modification of its active site, but sporidesmon has no affect on the other thiol disulfide oxidoreductases, GR, TR, or Trx (U. Srinivasan et al. 2006). The current observation of inhibition of Grx in L-DOPA treated cells with no change in its cellular content led us to predict a mechanism of inactivation analogous to sporidesmon adduction, involving covalent modification of Cys 22. Indeed, pre-incubation of Grx with oxidized L-DOPA gave concentration and time-dependent decreases in activity which followed pseudo-first order kinetics indicative of irreversible inactivation (Figure 2-11 A, B, p 62). Unlike Grx, GR was not inactivated appreciably by oxidized L-DOPA (Figure 2-11 C, p 62), consistent with retention of its activity in SHSY5Y cells treated with L-DOPA (Figure 2-8, p 57).

The thiol moiety of Cys22 at the active site of Grx1 has a remarkably low pKa of 3.5 due to its local environment (U. Srinivasan et al. 1997; S. C. Jao et al. 2006; J. J. Mieyal et al. 2008), making it much more reactive with thiol modifying agents. GSH and
typical protein cysteine residues have thiol pKa values around 8-9, so their thiol groups remain protonated and less nucleophilic at physiological pH. The nucleophilic reactivity of the Grx active site thiolate likely accounts for the selective inactivation of Grx via Michael addition of quinone, i.e., oxidized L-DOPA. Consistent with these interpretations, mass spectrometric results (Figure 2-12, p 66) document dopaquinone adduction of Grx on Cys 22. Although a recent proteomic study of isolated rat mitochondria treated with $[^{14}\text{C}]$ dopamine revealed other proteins which contain radiolabel indicative of quinone adduction (Laar Van, V et al. 2009), functional consequences were not examined so it is unknown whether modification of these proteins might also contribute to cytotoxicity. Furthermore, knockdown of Grx by ~50% using siRNA (Figure 2-13, p 67) led to increased apoptosis consistent with a requirement for Grx to maintain cell viability.

**Implications of Grx inactivation:** Grx is implicated in regulation of multiple cell signaling pathways that mediate apoptosis, cell growth, and cell survival (P. Klatt et al. 1999a; P. Klatt et al. 1999b; J. Wang et al. 2001; J. Wang et al. 2003; S. Qanungo et al. 2007). Hence, alterations in its deglutathionylase activity result in detrimental outcomes in many disease models (J. J. Mieyal et al. 2008). In particular, studies with Parkinson’s disease show Grx1 is important for maintenance of the mitochondrial permeability transition (U. Saeed et al. 2008) and protection from MPTP-induced mitochondrial dysfunction (R. S. Kenchappa et al. 2004). With quinone inactivation of Grx (shown here), the cells lose the protective mechanism of deglutathionylation and apoptotic cell death ensues.
**The Trx System:** The Trx system is responsible for reduction of intra- and inter-molecular disulfides and sulfenic acids ([Scheme 1-1], p 6). Isolated Trx was not inactivated by oxidized L-DOPA ([Figure 2-9 A], p 58). Consistent with this result, Trx activity was unaffected also in cells where GSH was depleted and low concentrations of L-DOPA led to apoptosis. At higher concentrations of L-DOPA in cells with replete GSH, dose-dependent loss of Trx activity was observed ([Figure 2-9, p 58]). The latter result may reflect glutathionylation of Trx protein on Cys residues separate from the active site, as reported previously (S. Casagrande et al. 2002), which could target it for degradation. However, the current observations dissociate changes in Trx activity from the observed pattern of changes in L-DOPA-induced apoptosis.

Unlike the results for Trx, TR exhibits two different patterns of loss of activity in cells treated with L-DOPA depending on the GSH status, perhaps reflecting different modes of regulation of its activity under different redox conditions. TR shows a parallel decrease in content and activity in response to L-DOPA treatment in neurons with replete GSH, similar to Trx. This observation is consistent with the lack of adductive deactivation of the isolated TR enzyme by oxidized L-DOPA, since the remaining TR protein obtained from the cells was fully active. However, co-treatment with BSO (depleting GSH) and L-DOPA led to loss of TR activity with no change in content ([Figure 2-9, p 58]). These results are in contrast with studies of the isolated enzyme, where no inhibition was observed ([Figure 2-11, p 62]) after preincubation with concentrations of oxidized L-DOPA that certainly exceed what would be generated in the SHSY5Y cells and what would occur in PD patients. Hence it is highly unlikely that quinone adduction of TR could explain its loss of activity in cells.
Several alternative considerations might explain the paradoxical data for TR. In the case where the decrease in TR activity corresponds to a decrease in content, this suggests that a modification of the TR targets it for degradation under conditions of replete GSH. For example, glutathionylation of TR is a likely oxidative modification under L-DOPA-induced oxidative stress when GSH is abundant and deglutathionylation by Grx is deactivated. In cells with depleted GSH, glutathionylation becomes less likely, so that irreversible oxidation at the TR active site such as formation of sulfenic and sulfonic acids may result in loss of activity without a signal for protease degradation.

Alternatively, diminution of GSH could elicit a cell wide antioxidant response leading to upregulation of TR. This response may not be elicited in cells with replete GSH. Since the half life of TR has been reported to be about 10 hr, this may be a likely explanation (A. Gallegos et al. 1997). Upregulation of content would have to be followed by an inhibitory modification which does not elicit proteosomal degradation of TR. Thus, loss of TR activity without loss of content might be attributed to coincidental and balancing increases in both synthesis and degradation of TR, with loss of activity due to protein modification other than quinone adduction.

Thirdly, TR may be inhibited by increased free metal ion concentration in the cells in response to L-DOPA treatment. Indeed, treatment of animals (rats and mice respectively) with 6-OHDA leads to iron (Fe2+, Fe3+) release from stores, which may contribute to ROS generation and oxidant-induced cell death (G. G. Borisenko et al. 2000; J. Wang et al. 2004). With the absence of GSH, iron ions have a decreased ability to be chelated and thus can bind to the TR active site thiols resulting in inhibition without concomitant degradation. Indeed, TR is known to be highly sensitive to inactivation by
divalent metal ions, so that its activity is assayed in the presence of EDTA (E. Martinez-Galisteo et al. 1992).

Regardless of the specific mechanism, loss of TR activity may compromise cell viability. Consistent with this interpretation, inhibition of TR by auranofin increased cell death (Figure 2-14, p 69).

**Cellular Implications for Inhibition of the Trx system:** TR is required for maintenance of the Trx system which reduces intra- and intermolecular disulfide bonds, and Trx has been observed in a number of studies to be neuroprotective including cell culture studies showing protection from MPP+ treatment (H. Masutani et al. 2004). Loss of TR not only impedes reduction of certain oxidative thiol modifications, it also activates cell signaling pathways that mediate cell death (A. K. Rundlof, E. S. Arner 2004). Thus, inhibition of either the Trx or Grx systems or both leads to SHSY5Y neuronal cell death. Comparison of the effects on apoptosis of ~50% inhibition of TR by auranofin or ~50% knockdown of Grx to the effects of L-DOPA treatment suggest that impairment of the two enzyme systems contributes in an additive fashion to the L-DOPA induced apoptosis (Figures 2-5, 2-7, 2-9, 2-13, 2-14, p 52, 55, 58, 67, 69).

2.5 Conclusions:

L-DOPA treatment of neuronal cells induces cell death in a concentration-dependent fashion that is potentiated in GSH deficient cells. L-DOPA leads to inactivation of Grx both in isolation and in neuronal cells. This inactivation appears to occur through irreversible quinone adduction of the enzyme active site. Furthermore, L-DOPA
decreases TR and Trx content which adds to the overall disruption of sulfhydryl homeostasis. The net result is increased apoptotic death of the SHSY5Y neurons. Both the Grx and Trx systems have been implicated in regulation of key intermediates in apoptotic signaling cascades, so inhibition of either or both of these enzyme systems impedes cell survival.

Further studies to delineate how each of these two enzyme systems alter specific cell signaling mechanisms leading to increased neuronal cell death would be beneficial in devising approaches to counteract the potential adverse affects of L-DOPA treatment in PD patients (see Chapter 3).

2.6 Materials and Methods:

Materials: CysteinyI-glutathione mixed disulfide was purchased from Toronto Research Chemicals. NADPH was purchased from Roche. TR, GR, and Trx were purchased from Sigma. Plasmid DNA (pET-24d, Novagen) encoding human Grx1 was prepared as described by Chrestensen et al. 1995 (C. A. Chrestensen et al. 1995). siRNA SMARTpool was purchased from Dharmacoon. Hoechst 33342 trihydrochloride and cell culturing supplies were purchased from Invitrogen. All other reagent grade chemicals were purchased from Sigma.

Synthesis of $[^3]H$ BSA-SS-Glutathione Mixed Disulfide Substrate: This radiolabeled prototype substrate for Grx was prepared as described previously (C. A. Chrestensen et al. 2000), except that $[^3]H$ GSH was substituted for $[^35]S$ GSH to form the final product.
Briefly, BSA was treated with excess IAM (iodoacetamide) (10 fold excess of protein) for 1 hour to block any free sulphydryls. SPDP (N-succinimidyl 3-(2-pyridyldithio) propionate) was used to form an amide bond on the N terminus of BSA and introduce a readily accessible glutathionylation site. This reaction was quenched with excess glycine. Upon removal of all small molecules by gel filtration, the modified BSA-SS-pyridine was treated with 10 fold excess $[^3]$H GSH overnight. $[^3]$H BSA-SSG was isolated through gel filtration and determination of its specific radioactivity documented near 1:1 stoichiometry of glutathionyl moiety and BSA protein.

**Cell Culture:** PC12 cells and SHSY5Y cells were obtained from the ATCC. Cells were maintained under an atmosphere of air / 5% CO2 at 37°C. PC12 cells were cultured in F12K media (Cell Gro) with 10% horse serum, 5% certified FBS, 2mM L-Glutamine, 100 Units/mL penicillin and 100µg/mL streptomycin. SHSY5Y medium was comprised of a 1:1 mixture of MEM and Hams F12 media (Mediatech, Cell Gro), containing 0.01mM Na pyruvate, 1 µM nonessential amino acids, and 10% certified fetal bovine serum. Cells were plated in 96-well plates (MTT assay), 6-well plates (Hoechst staining), or 100mm dishes and allowed to come to ~75% confluence prior to treatment.

**Treatments of cell in culture:** L-DOPA was added to cell culture media for 24 hr at concentrations ranging from 0-1mM.
Treatment with 0.1 mM BSO (buthionine sulfoximine) for 24 hr resulted in approximately 80% diminution of GSH, within the range of GSH loss in PD patients. This concentration of BSO (added concurrently with L-DOPA) was used for the experiments reported under Results.

Auranofin, chemical inhibitor of TR, was added to culture media at various concentrations for 30 min. After 30 min, the medium was replaced with fresh medium without auranofin and cells were either harvested immediately for measurements of TR activity or maintained for 24 hr for measurements of chromatin condensation by Hoechst staining.

Etoposide was added to cell culture media for 48 hrs at 10 μM. This was used as a positive control for chromatin condensation.

*Isolation / purification of Grx:* Grx was overexpressed in *E. coli* via a plasmid DNA construct, and purified to homogeneity (specific activity ~ 100 units/mg) as described in Jao *et al* (S. C. Jao *et al.* 2006).

*Treatment of Purified Thiol Disulfide Oxidoreductase Enzymes with Oxidized L-DOPA:* L-DOPA was dissolved in 1xPBS and allowed to oxidize overnight while exposed to air at 37°C. Oxidation of L-DOPA produces a variety of compounds as characterized spectrophotometrically in Asanuma *et al.* including dopaquinone, leukodopachrome, and dopachrome (M. Asanuma *et al.* 2004). Enzyme was incubated with various
concentrations of L-DOPA or 1x phosphate buffered saline (1xPBS) (control) at 30°C over the course of an hour. At various time aliquots of enzyme were removed and their respective activities assayed (see below). Inhibition was expressed as a percentage of activity lost based compared to the control.

Grx Activity: Our standard spectrophotometric coupled enzymatic assay was performed as described in (M. M. Gallogly, J. J. Mieyal 2007). Grx activity, (formation of GSSG from CSSG) corresponding to micromoles of NADPH oxidized per minute, was calculated using the extinction coefficient for NADPH (ε=6.2mM⁻¹cm⁻¹), along with correction factors to normalize plate reader values to standard spectrophotometer readings (C. A. Chrestensen et al. 2000). Alternatively the radiolabel assay which monitors Grx-catalyzed release of radiolabeled GSSG from [³H] BSA-SSG (C. A. Chrestensen et al. 2000) was used (see below).

Trx Activity: A spectrophotometric coupled enzymatic assay was performed as described in (D. W. Starke et al. 1997). Reaction mixtures containing Na/K phosphate buffer (0.1mM, pH 7.5), NADPH (0.2mM), thioredoxin reductase (TR) (0.09 U), and Trx (0.24 U) were prepared in individual wells of a 96 well plate to a final volume of 200μL and incubated for 5 min at 30°C. Care was taken to ensure that TR, as the coupling enzyme, was not limiting. Reactions were initiated with the addition of 2mM HEDS, and NADPH oxidation, corresponding to reduction of Trx-S₂, was monitored at 340 nm for 5 min. Units of Trx activity were calculated analogously to Grx activity (above).
Thioredoxin Reductase Activity: TR activity was measured by observation of NADPH-dependent reduction of 5,5’-dithiobis (2-nitrobenzoic acid) (DTNB) as adapted from a Sigma protocol. Briefly, NADPH (0.24mM), DTNB (5mM), BSA (0.2mg/mL), and EDTA (10mM) were combined in 100mM Na/K phosphate buffer pH 7.0 (final volume 600μL). TR was added to initiate the reaction which was monitored at A_{412nm} for 1 min. Activity was calculated in “DTNB units,” i.e. ΔA_{412nm}/min x (volume of assay/enzyme volume).

Glutathione Disulfide Reductase Activity: GR activity was measured spectrophotometrically, using an assay adapted from Carlberg and Mannervik (I. Carlberg, B. Mannervik 1985). 0.1M K phosphate buffer, 1mM EDTA pH 7.0, 0.1mM NADPH, and 1mM GSSG (final concentration) were incubated for 5 min at room temperature. GR was added to initiate the reaction (5.5 mU) and change in absorbance at 340nm was monitored for 5min. Enzymatic activity was calculated in an analogous fashion to Grx activity.

Cell Viability: Cell viability was measured according to reactivity of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide). MTT solution (0.5mg/mL final concentration) was placed on the cells for 2 hr at 37°C. Then an equal volume of MTT solubilization solution (0.1N HCl with anhydrous isopropanol and 10% Triton X) was added with shaking at room temperature for 1 hr to solubilize the formazan crystals. The absorbance reading at 570nm for each well was recorded, and corresponding nonspecific
absorbance at 690nm was subtracted according to manufacturer’s protocol. Net absorbance at 570nm corresponds to the amount of formazan formed by reduction of MTT by mitochondrial reductases, indicative of living cells and maintenance of their mitochondrial membrane potential.

*Cell Death Measurements:* Trypan blue exclusion was used to measure overall cell death. Trypan blue dye was mixed with an equal volume of cells. Those cells that took up the dye and turned blue, indicative of a compromised cellular membrane, were counted as dead.

*Apoptosis Measurements:* Hoechst 33342 trihydrochloride, used to analyze for chromatin condensation indicative of apoptosis, was applied to medium (final concentration 10µM) for 10 min at 37°C. Cells were counted on a Leica microscope with a DAPI fluorescent filter. Quantitative analysis of Hoechst staining of the cells was done in a blinded fashion relative to the respective treatments.

Caspase 3 activity was measured spectrofluorometrically. Treated cells were lysed in 1% Chaps, 150mM NaCl, 10mM EDTA, sigma protease inhibitor cocktail in 10mM HEPES pH 7.4. Cells were centrifuged for 30 min at 13,250 x g 4°C. Protein content was analyzed by BCA. Total protein was diluted to 10mg/mL. 100µg of protein was mixed in ICE buffer (50mM Tris-Cl pH7.2, 100mM KCl, 10% sucrose, 0.1% Chaps, 5mM DTT) to a total volume of 100µL. 1µL of 10mM caspase substrate (fluorogenic
substrate Ac-DEVD-AFC, Calbiochem) was added to the reaction and allowed to incubate 37°C for 1 hr. Fluorescence was measured with excitation of 360nm and emission of 460nm. Negative control used cell lysis buffer in place of cell lysate.

*Grx Activity in Cell Lysates:* Cells were collected in NP40 lysis buffer without protease inhibitors. The resulting cell lysates were analyzed immediately for GSH-dependent deglutathionylation of \([^3]H\) BSA-SSG as described previously (C. A. Chrestensen et al. 2000). 50 μg of cellular protein was incubated in a mixture of 0.25 mM GSH and 0.165 M Na K Pi buffer pH 7.5. After a 5 min incubation at 30°C, \([^3]H\) BSA-SSG (substrate) was added to the mixture to initiate the reaction. Aliquots of the reaction were taken out in a time dependent fashion and quenched with an equal volume of ice cold 20% TCA. Samples were centrifuged for 5min, 10,000xg at 4°C to pellet protein. Supernatant which contained released \([^3]H\) GSSG was measured by scintillation counting.

*Trx/ TR Activity in Cell Lysates:* Trx and TR activity were measured according to reduction of insulin disulfides and detection of the thiols by DTNB, as described in Arner et al. (E. S. Arner et al. 1999). SHSY5Y cells were ruptured in a lysis buffer comprised of 100mM NaCl, 20mM Tris pH 8, 0.5mM EDTA, 0.5% (v/v) NP-40, 1mM Na orthovanadate, 10mM Na pyrophosphate, and 20mM Na fluoride. 30μg of cell lysate (protein content) was incubated in a final volume of 50μL containing 0.3mM insulin, 0.66mM NADPH, 2.5mM EDTA, and 27mU TR or 0.24U Trx, respectively (depending on which enzyme was being assayed as the limiting factor) in 85mM HEPES, pH 7.6, for
40 minutes at 37°C. After incubation at 37°C, the reaction was stopped by the addition of 200μL of 8 M guanidine hydrochloride containing 1mM DTNB and absorbance at 405nm was read immediately. Separate experiments confirmed that these conditions were within the linear range of time- and concentration-dependence of the corresponding enzyme activities. Control measurements were made for each sample of cell lysate in the absence of additional enzyme. The control DTNB reactivity, corresponding to free thiols in the cell, was subtracted from values for lysates containing exogenous enzyme. Background absorbance, likely due to cellular thiols in the lysate, was subtracted and the net change in A405nm at 40 min was converted to specific enzyme activity (μmol thiol/min/mg) according to a standard curve for thiol (GSH) content generated under the same conditions.

**GR Activity in Cell Lysates:** Cell lysates (20-40μg) were added to a reaction mixture comprised of 0.1M K phosphate / 0.1mM EDTA, pH 7.0 (final concentration). 2mM NADPH dissolved in 10mM Tris HCl pH 7.0 was added to buffer to give a final concentration of 0.1mM. Reaction mixtures were incubated for 5 min at 37°C. Reactions were initiated by the addition of GSSG (final concentration 1mM). Loss of NADPH absorbance (340nm) was read continuously for 5 min to obtain the GR-mediated rate (D. W. Starke et al. 1997).

**Immunoblotting:** To determine relative content of specific proteins, 100μg (total protein) of lysate was loaded on SDS-PAGE gels (12.5%) and separated. Proteins were
transferred to polyvinylidene fluoride membranes (PVDF, Millipore Corp USA). Samples were probed with specific antibodies according to manufacturer instructions and relative content was determined by densitometric analysis (QuantityOne, Biorad) relative to the loading control (actin).

**Knockdown of Grx:** SHSY5Y cells were treated with lipofectamine 2000 and SMARTpool siRNA directed to several specific sequences of human Grx mRNA (Dharmacon). Knockdown was performed according to manufacturer’s protocol (Invitrogen). Lipofectamine 2000 and 140nM siRNA (final) was used to give ~ 50% knockdown of Grx. Control experiments involved transfection with scrambled siRNA sequences. Transfections were stopped 12 hr after initiation and cells were harvested 48 hr after transfection.

**Adduction of Grx by Oxidized L-DOPA and Mass Spectrometry:** Purified Grx (100μg) was incubated with oxidized L-DOPA or buffer for 1 hour. Inactivation of the enzyme was confirmed by the spectrophotometric assay. Grx samples were purified with C-18 ZipTips (Millipore) according to the manufacturer’s instructions with the exception that the samples were eluted with a larger volume of elution solution (50 μL). The samples were infused directly into the Applied Biosystems Q-STAR XL mass spectrometer in positive ion mode. For intact protein analysis, the mass spectra were deconvoluted using the BioAnalyst software. Trypsin (20μg/mL in 25mM ammonium bicarbonate) was used to digest Grx. Grx samples (treated with oxidized L-DOPA and untreated) were
incubated with trypsin (1:25 trypsin: protein) for 4 hr at 37°C. The MS/MS spectra were analyzed using MASCOT and assignments were confirmed by manual inspection. These experiments were carried out in collaboration with Dr. Anne Distler.
Chapter 3: Levodopa Activates Apoptosis Signaling Kinase 1 and Promotes Apoptosis in a Neuronal Model - Implications for Treatment of Parkinson’s Disease

This work was submitted in part for publication in *Molecular Pharmacology* (July 2010)

3.1 Abstract:

Parkinson’s disease (PD), the second most common neurodegenerative disease, is treated with chronic administration of Levodopa (L-DOPA). Paradoxically, L-DOPA induces cell death in cellular models of PD through increased oxidative stress. The mechanism appears to be disruption of sulfhydryl homeostasis as evidenced by loss of the thiol-disulfide oxidoreductase function of the glutaredoxin (Grx1) and thioredoxin (Trx1) enzyme systems (Sabens *et al.* 2010). Considering this loss of both Grx1 and Trx1 upon L-DOPA treatment, we sought to elucidate the mechanism(s) of L-DOPA induced apoptosis. Both the NFκB (nuclear factor κB) pathway and the ASK1 (apoptosis signaling kinase 1) pathway have been shown to be regulated by both Grx1 and Trx1 in other contexts, and both pathways have been implicated in cell death in PD model systems. Using the SHSY5Y cells as model neurons we found that NFκB activity was not altered by L-DOPA treatment. However, ASK1 was activated with L-DOPA treatment as indicated by phosphorylation of its downstream mitogen activated protein kinases (MAPK), p38 and JNK. Chemical inhibition of either p38 or JNK provided protection from L-DOPA induced apoptosis. In addition, knockdown of ASK1 protected
from L-DOPA induced neuronal cell death. These results identify ASK1 as the main pro-apoptotic pathway activated in response to L-DOPA treatment.
3.2 Introduction:

Parkinson’s disease (PD), the second most common neurodegenerative disease, affects primarily catecholaminergic neurons in the Substantia nigra of the brain (P. F. Riedlerer 2004). PD is linked to both genetic and environmental factors, involving oxidative stress, mitochondrial dysfunction, and protein aggregation (K. A. Maguire-Zeiss et al. 2005). Pathogenesis of PD manifests itself through loss of dopaminergic neurons resulting in bradykinesia, rigidity, resting tremor, and postural instability in patients. Many reports have attributed loss of dopaminergic neurons to the oxidative stress associated with increased dopamine turnover, deficient glutathione content, and increased iron content in the substantia nigra (C. W. Olanow, W. G. Tatton 1999; K. A. Maguire-Zeiss et al. 2005; S. Gandhi, N. W. Wood 2005). The primary agent for therapy of PD is Levodopa (L-DOPA), typically used in increasing doses as the disease progresses. Exposing catecholaminergic cells to increasing concentrations of L-DOPA has been reported to cause death in cultured cell lines (G. Walkinshaw, C. M. Waters 1995; E. A. Sabens et al. 2010). This effect is likely due to the pro-oxidant nature of L-DOPA.

L-DOPA is converted by decarboxylation to dopamine, which is reversibly oxidized to dopaquinone, generating superoxide and hydrogen peroxide. Dopaquinone also can irreversibly adduct cysteine residues on proteins, forming S-cysteinylnl dopamine adducts, thereby altering protein function (K. A. Maguire-Zeiss et al. 2005; E. A. Sabens et al. 2010). To anticipate improved therapy, the focus of the current study was to elucidate mechanisms by which treatment with the pro-oxidant L-DOPA may exacerbate
an already compromised cellular system and paradoxically aggravate a condition that it is meant to alleviate.

Oxidative stress in cells alters protein thiols by reversible oxidation either through formation of mixed disulfides with cellular glutathione (*i.e.*, protein-S-glutathionylation) or through formation of inter- and intra-molecular disulfides. Alternatively, protein thiols can be irreversibly oxidized to sulfinic and sulfonic acids. The two main enzyme systems which maintain sulfhydryl homeostasis are the glutaredoxin (Grx1) and thioredoxin (Trx1) systems. Grx1 specifically reduces glutathionylated proteins, and Trx1 specifically reduces intra- and inter-molecular disulfides (E. A. Sabens, J. J. Mieyal 2009). Both of these systems have been implicated in supporting cell survival (M. Saitoh *et al.* 1998; Y. Takagi *et al.* 2000; A. Mitsui *et al.* 2002; R. S. Kenchappa *et al.* 2002; R. S. Kenchappa, V. Ravindranath 2003; U. Saeed *et al.* 2008). We recently discovered that L-DOPA treatment of SHSY5Y neuronal cells leads to loss of activity of both Grx1 and Trx1, apparently through different mechanisms. Loss of these enzymatic activities due to direct enzyme modification and/or diminution in content is sufficient to induce apoptosis (E. A. Sabens *et al.* 2010); however, the cellular mechanism(s) of the pro-apoptotic response is currently unknown. Consequently, we investigated pathways involved in cell fate that have been previously shown to be regulated by both Grx1 and Trx1.

Activation of nuclear factor-kappa-B (NFκB), is typically considered a pro-survival pathway in cells; however, its role in neuronal cells is unresolved. Previous studies have implicated NFκB as a pro-apoptotic (H. Panet *et al.* 2001; V. Tarabin, M. Schwaninger 2004) as well as a pro-survival (H. J. Lee *et al.* 2001) transcription factor. These disparate results were obtained with the same model cell line (PC12). The
different outcomes might reflect different responses to the type or duration of stimulus for NFκB activation. NFκB normally exists in an inactive state, sequestered in the cytoplasm via non-covalent binding to IκB. Multiple stimuli, including oxidative stress (M. K. Meffert, D. Baltimore 2005), result in IκB kinase (IKK) activation and subsequent phosphorylation of IκB. NFκB is released and translocates to the nucleus. Evidence for upregulation of NFκB in post mortem brain samples from PD patients suggests such activation of NFκB is relevant to PD (A. Ghosh et al. 2007).

Redox regulation of this pathway occurs upstream of NFκB as well as directly on NFκB. Targets for redox regulation by Trx1 include regulation of release of NFκB from IκB and reduction of oxidized NFκB, allowing for DNA binding (reviewed in (H. Nakamura et al. 1997; J. Nordberg, E. S. Arner 2001)). Also, Grx1 regulates both DNA binding of NFκB (S. Qanungo et al. 2007) and activation of IKK, allowing for its phosphorylation of IκB (M. D. Shelton et al. 2009).

ASK1 is a central mediator of another pathway to apoptosis. Thus, activation of this MAPKKK (mitogen activated protein kinase kinase kinase) results in phosphorylation of downstream substrates such as p38-MAPK or c-Jun N terminal kinase (JNK), both implicated in propagation of apoptosis (S. J. Harper, N. Wilkie 2003; I. G. Onyango et al. 2005; J. Pan et al. 2007; M. Miloso et al. 2008; W. Yang et al. 2009). A wide variety of signaling molecules activate this pathway, including growth factors, UV radiation, cytokines, and oxidative stress (K. Takeda et al. 2008) (Scheme 1-6, p 37). In addition, the activity of ASK1 is reported to be modulated by a variety of negative and positive effector proteins, forming the so-called signalosome (see Scheme 3-1, p 92).
Many of these ASK1 modulators are sensitive to oxidative stress, including Grx1 and Trx1 themselves (A. Matsuzawa, H. Ichijo 2008).

ASK1 activation has been reported in several PD models. For example, 6-hydroxydopamine-treated rats displayed a loss of nigro-striatal neurons due to activation of the ASK1-JNK pathway (J. Pan et al. 2007). Similar treatment of SHSY5Y neuronal cells in culture led to phosphorylation of downstream kinases, JNK and p38, and ultimately to cell death, which could be prevented by knockdown of ASK1 (M. Ouyang, X. Shen 2006). Paraquat treatment also induces apoptosis through ASK1 activation, an event inhibited by overexpression of Trx1 (M. Niso-Santano et al. 2010). Treatment of male but not female mice with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) showed ASK1 activation leading to loss of dopaminergic neurons (S. Karunakaran et al. 2007a). The authors hypothesized this difference was due to the abundance of redox regulating enzymes, especially Grx1 and Trx1, in the female brain (U. Saeed et al. 2008).

In this study, we examined the effect of L-DOPA treatment on the NFκB and ASK1 pathways. Little effect was seen on transcriptional activity of NFκB. In contrast, we found that ASK1 is activated in SHSY5Y neurons by L-DOPA treatment, leading to phosphorylation of the downstream p38 and JNK MAPKs. Moreover, selective knockdown of ASK1 protected the SHSY5Y neurons from L-DOPA induced cell death.
On the left, ASK1 is represented in its inactive form in the cytosol, bound to some of its multiple negative regulators. Upon oxidative stimulus (L-DOPA treatment), these negative regulators may become oxidized or otherwise modified (e.g., dopaquinone adduction) and dissociate from ASK1. Concomitantly, ASK1 would be activated (autophosphorylated) and initiate a cascade of phosphorylation of downstream mediators, which induce apoptosis (shown at the right). L-DOPA treatment has been shown to cause loss of Grx1 and Trx1 activities (Sabens et al. 2010), which may impede reduction of oxidized negative regulators of ASK1, resulting in prolonged ASK1 activation. Details of this model, including the potential involvement of Daxx and DJ-1, are presented in the Discussion.
3.3 Results:

L-DOPA induced cell death was reported previously to be due to increased oxidative stress (G. Walkinshaw, C. M. Waters 1995). We recently found that the Grx1 and Trx1 systems were impaired by L-DOPA treatment of SHSY5Y neurons, leading to cell death (E. A. Sabens et al. 2010) (Chapter 2). To further elucidate the mechanism of L-DOPA induced apoptosis, we examined signaling pathways known to be regulated by both Trx1 and Grx1 and potentially involved in neuronal cell death.

*L-DOPA Treatment does not Alter NFκB Activity:* NFκB has been reported to be regulated by both Grx1 (S. Qanungo et al. 2007; M. D. Shelton et al. 2009) and Trx1 (reviewed in (J. Nordberg, E. S. Arner 2001)). Furthermore, in catecholaminergic PC12 cells and in mouse brain tissue NFκB has been interpreted to mediate both pro-survival (G. Taglialatela et al. 1997; H. J. Lee et al. 2001) and pro-apoptotic (H. Panet et al. 2001; V. Tarabin, M. Schwaninger 2004; A. Ghosh et al. 2007) outcomes, suggesting regulation and function of NFκB in neuronal cells may be stimulus dependent. We found NFκB transcriptional activity to be essentially unchanged after L-DOPA treatment (Figure 3-1, p 94). This result eliminates the NFκB pathway as a contributor to L-DOPA induced apoptosis in the SHSY5Y neurons.
**Figure 3-1:** NFκB Transcriptional Activity is Not Altered by L-DOPA Treatment of SHSY5Y Cells

Bar graph representing NFκB luciferase activity relative to Renilla luciferase activity, which served as the measure of transfection efficiency in each case. Values are represented as fold increase relative to control (untreated), with the control normalized to 1 (white bar). L-DOPA (gray bars) or TNFα (50 ng/mL, used as a positive control, black bar), were added 24 hr post transfection. Samples were analyzed 24 hr after treatment. n=3 (biological replicates). Three separate preparations of cells were treated and analyzed for each condition, and the analysis of each set of cells was done at least two times.
**L-DOPA Treatment of SHSY5Y Cells leads to Increased Phosphorylation of MAPK:** We then turned our attention to ASK1, another central intermediate involved in apoptosis. ASK1 has been shown to be regulated by both the Grx1 (J. J. Song, Y. J. Lee 2003) and Trx1 (M. Saitoh et al. 1998) systems in other contexts. We first examined downstream targets of ASK1, JNK and p38. Both of these kinases initiate apoptotic cascades when phosphorylated by ASK1 (S. J. Harper, N. Wilkie 2003; I. G. Onyango et al. 2005; W. Yang et al. 2009). We found that L-DOPA treatment increased phosphorylation of both p38 (Figure 3-2 A, p 96) and JNK (Figure 3-2 B, p 96) in a dose-dependent manner. Quantification showed that increases in phosphorylation were statistically significant (Figures 3-2 C and 3-2 D, p 96). This increased phosphorylation led us to examine whether these kinases might be mediators of L-DOPA induced apoptotic cell death. Accordingly, we used chemical inhibitors to block the function of p38 and JNK. SB202190, a selective p38 inhibitor, was applied to cells in a concentration range (0-10 μM) that has been reported to be selective for p38 (α and β). Under these conditions, a decrease in overall L-DOPA-induced cell death was observed compared to control (Figure 3-3, p 98). Phase contrast images, to distinguish between dividing cells and dying cells, confirmed that cell division did not interfere with the assessment of apoptosis (Figure 3-3 A, p 98). L-DOPA treatment increased apoptosis from < 10% to > 40%. In the presence of 5 μM p38 inhibitor, apoptosis was diminished to near control levels, indicating the importance of this pathway in L-DOPA induced apoptosis.
Figure 3-2: Increased Phosphorylation of p38 and JNK Occurs with L-DOPA Treatment of SHSY5Y Cells
(A) Representative western blot showing increased phosphorylation of p38. SHSY5Y cells were treated for 24 hr with the indicated concentrations of L-DOPA, and control cells were incubated in parallel. (B) Representative western blot showing increased phosphorylation of JNK. SHSY5Y cells were treated for 24 hr with the indicated concentrations of L-DOPA, and control cells were incubated in parallel. Two bands are observed for JNK, representing the isoforms present within SHSY5Y cells (K. Mielke et al. 2000). (C) Bar graph displaying quantification of multiple western blots measuring phosphorylation of p38. Each was quantified by densitometry and normalized to total p38, which was developed and quantified on the same western blot. n=4 (biological replicates. * p<0.01 compared to control. (p=.18 for 250μM compared to 100μM) (D) Bar graph displaying quantification of western blots measuring phosphorylation of JNK. Each value was normalized to total protein. n=4 (biological replicates). # p<0.05 compared to control. (p=.03 for 250μM compared to 100μM).
Figure 3-3: Inhibition of p38 Leads to Decreased Apoptosis in L-DOPA treated SHSY5Y Cells
(A) Hoechst images taken on a Leica microscope at 20x magnification. Upper left panels display control cells, not exposed to L-DOPA or the p38 inhibitor (DAPI filter and phase contrast images). Lower left panels display analogous images for cells treated with 100 μM L-DOPA without p38 inhibitor. The upper and lower corresponding panels at the right display cells that were first exposed to 5μM p38 Inhibitor (SB202190) for 30 min prior to treatment with vehicle (PBS) or 100μM L-DOPA, respectively. Cells were imaged 24 hr after L-DOPA treatment. (B) Bar graph representing quantification of SHSY5Y cells for chromatin condensation following 24 hr L-DOPA treatment. Cells were treated 30 min prior to L-DOPA with p38 inhibitor (SB202190) or DMSO (vehicle). n=3. Three separate preparations of cells were treated and analyzed for each condition, and the analysis of each set of cells was done three times. # represents p<0.05 compared to control, * p<0.01 compared to control, @ p<0.01 compared to L-DOPA. (C) Bar graph representing cell viability as measured through MTT reduction. Cells were treated with the p38 inhibitor (SB202190) for 30 min prior to 24 hr L-DOPA treatment. n=5 (biological replicates). ξ p<0.05 compared to L-DOPA, *p<0.01 compared to control. Each set of data was normalized to its respective non-treated control, where raw values in absorbance units (AU) are Control: 0.24±0.02 AU, 100μM L-DOPA: 0.07±0.01 AU, 2.5μM SB202190: 0.26 ±0.04 AU, 2.5μM SB202190 + 100μM L-DOPA: 0.11±0.03 AU, 5μM SB202190: 0.21±0.01 AU, 5μM SB202190 + 100μM L-DOPA: 0.11±0.01 AU.
Figure 3-4: Inhibition of JNK Prevents L-DOPA Induced Apoptosis

(A) Untreated

No Inhibitor

10 μM JNK Inhibitor

(B) Percent Apoptosis

(C) Percent Viability

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<tr>
<th>JNK Inhibitor</th>
<th>100 μM L-DOPA</th>
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(A) Hoechst images taken on Leica microscope at 20x magnification. Left hand side contains control cells, not exposed to drug. On left side cells were exposed to 10 μM JNK inhibitor (SP600125) for 30 min prior to treatment with vehicle (PBS) or 100μM L-DOPA. Cells images were taken 24 hr after L-DOPA treatment. (B) Bar graph representing chromatin condensation of SHSY5Y cells following 24 hr L-DOPA treatment. Cells were treated 30 min prior to L-DOPA with 10μM JNK Inhibitor (SP600125) or DMSO (vehicle). n=3. Three separate preparations of cells were treated and analyzed for each condition, and the analysis of each set of cells was done three times. # represents p<0.05 compared to control, * represents p<0.01 compared to control, @ p<0.01 compared to L-DOPA. (C) Bar graph representing cell viability as measured through MTT reduction. Cells were treated with the JNK inhibitor (SP600125) for 30 min prior to 24 hr L-DOPA treatment. n=5 (biological replicates). ξ p< 0.05 compared to LDOPA * p<0.01 compared to control. # p<0.05 compared to control. Each set of data was normalized to its respective non-treated control, where raw values in absorbance units (AU) are Control: 0.24±0.02 AU, 100μM L-DOPA: 0.07±0.01 AU, 5μM SP600125: 0.199±0.00 AU, 5μM SP600125 + 100μM L-DOPA: 0.06±0.00 AU, 10μM SP600125: 0.18±0.01 AU, 10μM SP600125 + 100μM L-DOPA: 0.10 ±0.01 AU.
As further confirmation, the MTT reduction assay also reflected an increase in cell viability upon inhibition of p38 compared to L-DOPA treatment alone (Figure 3-3 C, p 98).

SP600125, a pan JNK inhibitor was used to test the involvement of JNK. We observed a small but significant decrease in overall L-DOPA induced cell death compared to control cells at a dose of 5 µM. Doubling the dose of inhibitor afforded somewhat greater protection, but cell death was not diminished to control levels (Figures 3-4 A and 3-4 B, p 100). We interpret these results to mean that inhibition of JNK alone is not sufficient to protect from L-DOPA induced cell death. These results were corroborated by MTT analysis where inhibition of JNK was observed to increase cell viability compared to L-DOPA treatment alone (Figure 3-4 C, p 100).

Since inhibition of either of the downstream MAPKs, p38 or JNK, offered protection from L-DOPA induced apoptosis, we next examined coincident treatment with both inhibitors to detect if there was synergism. Maximum protection against L-DOPA induced apoptosis was observed at 5µM of the p38 inhibitor and at 10µM of the JNK inhibitor. Combinations of lower concentrations of the two inhibitors were therefore used to investigate synergism. The inhibitors offered no protection when each was added separately at low concentration (p38 inhibitor at 2.5 µM and JNK inhibitor at 5 µM, Figures 3-3 and 3-4, p 98, 100). In contrast, addition of both inhibitors (each at 2.5 µM) offered significant protection from L-DOPA induced apoptosis (Figures 3-5 A and 3-5 B, p 103). Thus, the inhibitors of p38 and JNK kinases act in a synergistic manner, indicating that both contribute importantly to mediating the apoptotic response.
Figure 3-5: Dual Inhibition of p38 and JNK Results in Decreased Apoptosis in L-DOPA Treated SHSY5Y Cells
(A) Hoechst images taken on Leica microscope at 20x magnification. Left hand side shows control cells, not exposed to drug. Shown on the right are cells that were exposed to 2.5 μM JNK inhibitor (SP600125) and 2.5μM p38 inhibitor (SB202190) for 30 min prior to treatment with vehicle (PBS) or 100μM L-DOPA. Cells were imaged 24 hr after L-DOPA treatment. (B) Bar graph of quantification of Hoechst staining. White bars represent samples without inhibitor treatment, light gray bars represent samples treated with 2.5μM p38 inhibitor and 2.5μM JNK inhibitor, black bars represent samples treated with 2.5μM p38 inhibitor and 5μM JNK inhibitor, and dark gray bars represent samples treated with 5μM p38 inhibitor and 5μM JNK inhibitor. Samples were analyzed in a blinded fashion, n=3. Three separate preparations of cells were treated and analyzed for each condition, and the analysis of each set of cells was done three times. * represents p<0.01 compared to control, @ p<0.01 compared to L-DOPA. (C) Bar graph showing increased cell viability (MTT assay) under L-DOPA treatment conditions when the p38 and JNK inhibitors are added. White bars represent control (no inhibitors), light gray bars represent 2.5μM p38 inhibitor (SB202190) and 2.5μM JNK inhibitor (SP600125), black bars represent samples treated with 2.5μM p38 inhibitor and 5μM JNK inhibitor, and dark gray bars represent 5μM p38 inhibitor (SB202190) and 5μM JNK inhibitor (SP600125). Each set of data was normalized to its respective non-treated control, where raw values in absorbance units (AU) are: Control 0.24 ± 0.02 AU, 100μM L-DOPA: 0.07 ± 0.01 AU, 2.5μM SB202190/2.5μM SP600125: 0.37 ± 0.02 AU, 2.5μM SB202190/2.5μM SP600125 + 100μM L-DOPA: 0.26 ± 0.02 AU, 2.5μM SB202190/5μM SP600125: 0.39 ± 0.04 AU, 2.5μM SB202190/5μM SP600125 + 100μM L-DOPA: 0.27 ± 0.04 AU, 5μM SB202190/5μM SP600125: 0.21 ± 0.01 AU, 5μM SB202190/5μM SP600125 + 100μM L-DOPA: 0.15 ± 0.02 AU.
Figure 3-6: ASK1 Expression Is Diminished by 80% with Transient Transfection of siRNA in SHSY5Y Cells

(A) Knockdown of ASK1 was measured by western blot analysis prior to L-DOPA treatment (inset). ASK1 remained knocked down after the period of L-DOPA treatment as shown in representative western blot, top right, n=3 siASK1, n=2 siSCRM. (B) Bar graph quantifying ASK1 knockdown in SHSY5Y cells after treatment with siRNA for 24 hr, normalized to the actin loading control. The difference is significant at p<0.05 (n=3, biological replicates).
However, combination of the inhibitors at a higher concentration of each did not provide further protection. Analogous results were seen for changes in cell viability measured by MTT reduction (Figure 3-5 C, p 103).

Knockdown of ASK1 Prevents L-DOPA induced apoptosis:

To determine whether ASK1 is the primary kinase responsible for activation of p38 and JNK and the apoptotic response to L-DOPA we selectively knocked ASK1 down in the SHSY5Y neurons. We treated the cells with ASK1-targeted siRNA or scrambled siRNA for 24 hr and then further treated with L-DOPA for 24 hr. We confirmed that ASK1 levels were substantially diminished by the targeted siRNA at 24 hr just prior to L-DOPA treatment (Figure 3-6, p 105). Thus, ASK1 was knocked down ~ 77% compared to control cells. Upon treatment with L-DOPA (100μM), apoptosis in the ASK1-deficient cells was diminished to near control levels (Figures 3-7 A and 3-7 B, p 107). Since ASK1 was not fully knocked down, the small increase in apoptosis after L-DOPA treatment may be attributable to remaining ASK1; however, the possibility of contributions from other mediators, upstream of p38, cannot be excluded.

3.4 Discussion:

In our previous study (Chapter 2) we found that deactivation of the Grx and/or Trx enzyme systems is sufficient to induce apoptosis, and L-DOPA treatment impaired both systems (E. A. Sabens et al. 2010). Hence, using the SHSY5Y neuronal cell line as
**Figure 3-7:** Knockdown of ASK1 Prevents L-DOPA Induced Apoptosis

(A) Hoechst images were taken at 40x magnification. Representative images using a DAPI filter (left) or phase contrast (right) are shown. Top panel shows representative images of Hoechst staining of cells treated with scrambled siRNA (left) *versus* ASK1 knockdown cells (right) treated with vehicle (PBS). The bottom row shows images of cells after treatment with 100 μM L-DOPA.

(B) Bar graph showing quantification of percent apoptosis (n= 4, biological replicates). Four separate preparations of cells were treated and analyzed for each condition, and the analysis of each set of cells was done three times.
a model in this study we investigated pathways (NFκB and ASK1) involved in cell fate that have been previously shown to be regulated by both Grx1 and Trx1 in other contexts.

Activation or inactivation of NFκB was not observed (Figure 3-1, p 94). Instead, the observations support a scenario whereby L-DOPA initiates an apoptotic cascade through activation of the ASK1 pathway, causing phosphorylation of downstream p38 and JNK kinases (Figure 3-2, p 96). L-DOPA induced apoptosis was largely prevented by inhibition of either of these downstream kinases (Figure 3-3, 3-4, 3-5, p 98, 100, 103) or by knockdown of the ASK1 protein (Figure 3-7, p 107). The complex interactive signaling pathways within the cell which control apoptosis provide many points of initiation of a death cascade (O. A. Levy et al. 2009). Hence it is remarkable that knockdown of ASK1 or inhibition of its downstream molecular targets nearly abolished L-DOPA induced apoptosis. This conclusion is based on the assumption that all cells obtained a uniform knockdown of ASK1 by ~80% since transfection efficiency was not tested (e.g. by using a construct expressing GFP). Thus, the oxidative stress imposed by administration of L-DOPA promotes apoptosis by activation of ASK1 through the p38 and JNK branch of its signal transduction pathway.

Both p38 and JNK have been shown to be activated by oxidative stress in a number of other studies of cultured cells (D. S. Cassarino et al. 2000), animal models (J. Pan et al. 2007), and post mortem human brains (I. Ferrer et al. 2001; S. Hunot et al. 2004). For example, increased phosphorylation of p38 was found in post mortem samples of cortical neurons from patients with Lewy body dementia or Alzheimer’s disease compared to age matched controls (I. Ferrer et al. 2001). Knockout of JNK 3, the main isoform expressed in brain, protects mice and/or cortical derived neurons from a
variety of apoptotic insults including Aβ toxicity, oxygen deprivation, kainic acid treatment, and glucose deprivation (S. J. Harper, N. Wilkie 2003). Inhibition of JNK3 might therefore prevent further loss of dopaminergic neurons in PD patients, and agents targeted to the JNK3 activation pathway are currently in clinical trials. Unfortunately, progression of PD does not appear to be delayed in patients receiving a so-called “JNK3 inhibitor”. However, this inhibitor targets an upstream kinase, MLK (mixed lineage kinase), which activates JNK at the same level as ASK1 (The Parkinson Study Group PRECEPT Investigators 2007). According to the findings of this study ASK1 is likely the major upstream kinase responsible for JNK activation. Hence, targeting ASK1 may be a more effective therapeutic approach, especially if L-DOPA treatment promotes apoptosis through activation of ASK1 in the brains of PD patients.

In the cell model, L-DOPA induces an oxidative stress that may lead to direct alteration of ASK1 or to modification and inhibition of the various regulators of ASK1 (described below). Previous studies with the LNCaP prostate cell line showed that treatment of ASK1 with N-ethylmaleimide, a thiol modifying agent, activates ASK1 (J. V. Cross, D. J. Templeton 2004). In another study site-directed mutagenesis of Cys residues on ASK1 altered Trx1 binding and phosphorylation of JNK (P. J. Nadeau et al. 2009). Analogous phenomena might occur with L-DOPA treatment, where quinone adduction or other modifications of cysteine residues such as sulfenic acid formation and/or glutathionylation could lead to activation of ASK1. It is conceivable that such modifications may interfere with phosphorylation of ASK1 which in many cases are inactivating or lead to degradation (A. H. Kim et al. 2001; E. H. Goldman et al. 2004).
ASK1 exists as a signalosome, binding many positive and negative regulating proteins. Furthermore, scaffolding proteins such as JNK interacting proteins help to localize ASK1 with its downstream effector molecules (C. R. Weston, R. J. Davis 2007). It is proposed that dissociation of negative regulators and/or association of positive regulators leads to phosphorylation and activation of ASK1 (A. Matsuzawa, H. Ichijo 2008). Several negative regulators of ASK1 are oxidatively modified in other contexts, resulting in alteration in function. For example, heat shock proteins (HSP) 72 and 90 have been reported to regulate the oligomeric state and associated activity of ASK1 (H. S. Park et al. 2002; R. Zhang et al. 2005). HSP72 has been shown to be modified by 4-hydroxynonenal in hepatocytes leading to loss of ATPase function (D. L. Carbone et al. 2004). Additionally, HSP90 is reported to be inactivated by S-nitrosylation in endothelial cells. (A. Martinez-Ruiz et al. 2005). Peroxiredoxin 1 (Prx1), another negative regulator, binds at the Trx1 domain on ASK1 and prevents ASK1 activation (S. Y. Kim et al. 2008). Prx1 was found to be inactivated in MN9D cells after treatment with pro-oxidant 6-hydroxydopamine (Y. M. Lee et al. 2008). DJ-1, which functions as an antioxidant, transcriptional co-activator, and molecular chaperone, has been implicated in PD in connection with naturally occurring mutations of the protein. Also, DJ-1 is implicated as a direct binding positive regulator of ASK1 (J. Waak et al. 2009), or an indirect negative regulator of ASK1 by binding to Daxx (U. Saeed et al. 2010). Oxidation of DJ-1 alters these functions, and it was recently reported that knockdown of Grx1 exacerbates these effects on DJ-1 function (U. Saeed et al. 2010).

As described above, many potential ASK1-binding proteins are susceptible to oxidative cysteine modification and concomitant loss of function. Thus, the central role
of glutaredoxin and thioredoxin in thiol homeostasis links these enzyme systems broadly to regulation of ASK1 function. The L-DOPA induced activation of ASK1 and neuronal apoptosis reported in this study may therefore result from diminished Grx1 and/or Trx1 activity (E. A. Sabens et al. 2010), preventing the cell from maintaining proper thiol status of ASK1 or associated proteins. Other examples of this type of relationship have been reported. For example, cadmium treatment leads to activation of ASK1 and its downstream MAPK, JNK (S. D. Kim et al. 2005), and cadmium inhibits the components of the Grx1 and Trx1 systems (C. A. Chrestensen et al. 2000).

Besides their regulation of various other effectors of ASK1 activity, Grx1 and Trx1 themselves have been identified as negative regulators of ASK1. Grx1 binds to the C-terminus (J. J. Song, Y. J. Lee 2003) and Trx1 to the N-terminus (M. Saitoh et al. 1998) of ASK1, and each of them are dissociated upon oxidative modification (M. Saitoh et al. 1998; J. J. Song, Y. J. Lee 2003). Thus, L-DOPA treatment could lead to apoptosis simply through release of modified Grx1 and Trx1 from ASK1 (See Appendix) or via the impact of deactivation of these enzymes on the function of the other ASK1 effectors. Distinguishing among these possibilities is the object of future studies.

3.5 Conclusions:

Presented in this chapter, we show L-DOPA induction of apoptosis through activation of ASK1 signaling cascade. Inhibition of downstream targets, p38 and JNK, or knockdown of ASK1 is sufficient to prevent the majority of L-DOPA induced apoptosis indicting this MAPK cascade as the major pathway of apoptosis. This study provides a
clear mechanism of L-DOPA induced apoptosis, and a potential therapeutic target through inhibition of ASK1.

3.6 Materials and Methods:

Materials: SHSY5Y cells were obtained from ATCC (Manassas, VA). Hoechst 33342 trihydrochloride, lipofectamine 2000, and cell culture reagents were obtained from Invitrogen. The selective inhibitors, SB202190 targeted to p38 and SP600125 targeted to JNK, were obtained from Sigma. ASK1 antibodies, H-300 (rabbit anti-human for immunoprecipitation) and F-9 (mouse anti-human for immunoblotting), were purchased from Santa Cruz Biotechnologies. Anti-actin antibody (clone AC74) was purchased from Sigma. Antibodies for western blot detection of the MAPKs were kind gifts from Dr. Laura Nagy (Cleveland Clinic Foundation) and were obtained as follows: total JNK (Santa Cruz), phospho-JNK (Thr 183, Tyr 185) (Cell Signaling), total p38 (Santa Cruz Biotechnologies) and phospho-p38 (Thr 180, Tyr 182) (Promega). ASK1-targeted and nontargeted control SMARTPool siRNA samples were purchased from Dharmacon. Gradient gels (10-20%) were purchased from Bio-Rad. All other chemicals were purchased from Sigma unless otherwise specified.

Cell Culture: SHSY5Y cells (immortalized neuronal cell line derived from human neuroblastoma) were maintained under an atmosphere of air with 5% CO₂ at 37°C. The medium was comprised of a 1:1 mixture of MEM and Hams F12 media (Mediatech, Cell Gro), containing 0.01 mM Na pyruvate, 1 μM nonessential amino acids and 10%
certified fetal bovine serum. Cells were plated in 100 mm dishes, 96-well plates (for assay with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)) or 6-well plates (for Hoechst staining) and allowed to come to ~75% confluency prior to treatment.

*Treatments of cells in culture:* L-DOPA was added to cell culture media for 24 hr at concentrations ranging from 0 to 1 mM. For MAPK inhibitor studies, SB202190 (p38 inhibitor) or SP600125 (JNK1, 2, 3 inhibitor) were applied to cells 30 min before L-DOPA was added to the medium. Cells were incubated overnight and analyzed 24 hr post L-DOPA treatment.

*Cell Death Measurement:* To detect the chromatin condensation indicative of apoptosis, Hoechst 33342 trihydrochloride was added to the medium of each set of cells to a final concentration of 10 µM for 10 min at 37°C, according to manufacturer’s protocol. Cells were counted on a Leica microscope (CTR6500) with a DAPI fluorescent filter. Quantitative analysis of Hoechst staining of the cells was done in a blinded fashion with respect to treatment conditions.

*Cell Viability:* Cell viability was measured according to reactivity of MTT according to manufacturer’s protocol. MTT at a final concentration of 0.5mg/mL was placed on the cells for 3 hr at 37°C. Medium was removed and replaced with MTT solubilization
solution (0.1N HCl in anhydrous isopropanol and 10% Triton X). Culture dishes were shaken at room temperature for 1 hr to solubilize the formazan crystals before measuring the absorbance at 570 nm for each well. Nonspecific absorbance at 690 nm was subtracted.

**NFκB Luciferase Activity:** SHSY5Y cells were plated to 60% confluence and transfected for 12 hr with 1 μg of 5x NFκB luciferase plasmid (Stratagene, La Jolla, CA) and 0.1 μg of Renilla plasmid (Promega, Madison, WI) for 12 hr, according to the Lipofectamine (Invitrogen) reagent protocol. Transfection medium was then changed to replete medium and the cells incubated for an additional 12 hr at 37°C. Cells were then treated with L-DOPA for 24 hr. After harvesting, cells were assayed using the Dual-Luciferase assay system (Promega), measuring emission with a luminometer (SOFTmax PROsoftware, Molecular Devices, Sunnyvale, CA). NFκB activity is expressed as firefly luciferase dependent luminescence relative to Renilla luciferase dependent luminescence.

**Detection of Phosphorylated Proteins:** SHSY5Y cells were lysed in RIPA buffer containing 150 mM NaCl, 10 mM EDTA, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, and 50 mM Tris, pH 7.5, along with Sigma protease inhibitor cocktail. Protein determinations were performed using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). For western blots, 50-100 μg of total protein was run on a 10% SDS-PAGE gel before transfer to a PVDF membrane. Membranes were blocked with 3%
BSA solution and probed overnight with the appropriate phospho-protein antibody in a 1% BSA solution. Blots were developed using enhanced chemiluminescent substrate (Pierce). Blots were subsequently stripped with stripping buffer (20% SDS (w/v), 56 mM Tris HCl, 0.1 M BME, 1 mM DTT) for 30 min at 60°C and reprobed for total content of the target proteins in a similar fashion. Quantification of blots was performed on digitized gel scans with BioRad Quantity One Software. Estimates of phosphorylated proteins are presented as densitometric ratios, normalized to the corresponding total protein content.

**Knockdown of ASK1:** SmartPOOL siRNA (Dharmacon) was used to knockdown ASK1. 200 nM siRNA was transfected into cells with Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. Cells were incubated with siRNA for 6 hr in OptiMEM. Medium was changed to serum replete medium. Cells were treated with L-DOPA or vehicle 24 hr after transfection with siRNA. Cell death was measured 24 hr post L-DOPA treatment (48 hr post transfection with siRNA). Western blot analysis was used to confirm that ASK1 remained knocked down for the duration of L-DOPA treatment.
Chapter 4: Major Findings, Future Directions, and Preliminary Data.

4.1 Overview of Major Findings

L-DOPA treatment of neuronal cells induces cell death in a concentration-dependent fashion that is potentiated in GSH deficient cells. L-DOPA leads to inactivation of glutaredoxin (Grx) both with purified protein and in neuronal cells. This inactivation occurs through irreversible quinone adduction of the Grx enzyme active site as determined by mass spectrometry of purified enzyme (Figure 2-12, p 66). Furthermore, L-DOPA decreases TR1 and Trx1 content (Figure 2-9, p 58) which adds to the overall disruption of sulfhydryl homeostasis. Both the Grx and Trx enzyme systems are required for cell survival (Figure 2-13, 2-14, p 67, 69). The net result is increased apoptotic death of the SHSY5Y neurons. Both the Grx and Trx systems have been implicated in regulation of key intermediates in apoptotic signaling cascades, so inhibition of either or both of these enzyme systems impedes cell survival.

Increased cell death concomitant with loss of Trx and Grx indicates that these proteins regulate cell fate pathways, including the NFκB and ASK1 pathway. Both of these pathways have previously been shown to be dually regulated by Grx and Trx (M. Saitoh et al. 1998; K. Hirota et al. 2000; J. J. Song, Y. J. Lee 2003; S. Qanungo et al. 2007; M. D. Shelton et al. 2007). We found no change in transcriptional activity of NFκB (Figure 3-1, p 94) with L-DOPA treatment. Instead, we discovered activation of downstream targets of ASK1, p38 and JNK, with L-DOPA treatment (Figure 3-2, p 96). Chemical inhibition of either of these downstream targets resulted in protection from L-DOPA-induced cell death (Figure 3-3, 3-4, p 98, 100). Furthermore, knockdown of
ASK1 by RNAi resulted in complete protection from L-DOPA-induced cell death implicating this MAPKKK as the main activator of L-DOPA-induced apoptosis (Figure 3-6, 3-7, p 105, 107). We propose that this activation occurs through loss of Grx and/or Trx after L-DOPA treatment. Grx and Trx may interact directly with ASK1 through binding to its C and N terminus, respectively. Both Trx and Grx are negative regulators of ASK1 and require a reduced active site to maintain association with ASK1 (M. Saitoh et al. 1998; J. J. Song, Y. J. Lee 2003). Alternatively, Grx and Trx may catalyze reduction of oxidized protein modulators of ASK1, so that loss of these two enzymes may result in irreversible oxidation of negative regulators of ASK1 as discussed in Chapter 3 (see Scheme 3-1, p 92). Furthermore, DJ-1 binds Daxx in the nucleus preventing binding of this positive effector of ASK1 and activation of ASK1 by Daxx. DJ-1 has been shown to be oxidized resulting in the release of Daxx. Loss of Grx and/or Trx function may result in sustained oxidized DJ-1 (S. Karunakaran et al. 2007a). Despite the unknown location of regulation by Grx and Trx, we show ASK1 activation as the major apoptotic mechanism with L-DOPA treatment of SHSY5Y neurons, and future studies will address the site(s) of regulation by Grx and/or Trx (as discussed below).

4.2 Significance of Findings

The findings presented in this thesis show a mechanism by which L-DOPA therapy may be detrimental to PD patients. The increase in oxidative stress and reactive quinones provided by L-DOPA treatment leads to activation of a single, major apoptotic pathway through activation of ASK1. Future studies examining the activation of ASK1 in a more complex model system i.e. mouse model (discussed below) would corroborate these
important findings. ASK1 would then become a new therapeutic target to enable co-therapies with L-DOPA preventing its unwanted toxicity to dopaminergic neurons. Since previous antioxidant therapies in patients have not shown statistical differences in prognosis (The Parkinson Study Group PRECEPT Investigators 2007), targeting ASK1 may provide added benefit preserving the dopaminergic neurons that remain in the diagnosed patient. Targeting ASK1 and diminishing the amount of dopaminergic neuronal death would significantly improve patient quality of life and possibly duration. Furthermore, these studies may prove to be widespread in many oxidative stress diseases including cardiovascular disease, other neurodegenerative diseases, and diabetes, where ASK1 activation leads to increased apoptosis, making an inhibitor of ASK1 a potential therapy for numerous diseases.

4.3 Future Directions:

Elucidation of the Mechanism(s) of Loss of TR and Trx with L-DOPA Treatment of SHSY5Y neurons: We observed coincident loss of content and activity of TR and Trx after L-DOPA treatment under normal cellular content of GSH. This concurrence suggests either increased degradation of the proteins or decreased production of the proteins or both. Measurements of mRNA levels of TR and Trx over a time course of L-DOPA treatment versus control conditions would reveal whether the rate of production of these two proteins changed. Decreased production might occur through diminution of activating transcription factors for these two proteins. However, under oxidative stress conditions, the cell would be expected to initiate an antioxidant response resulting in increased production of Trx and TR. Indeed, ARE and Nrf2 elements, which have been
proposed to control Trx and TR transcription, are upregulated in response to oxidative stress (Y. Taniguchi et al. 1996; Z. R. Stoytcheva, M. J. Berry 2009). Therefore, we hypothesize that L-DOPA treatment would not decrease production of the proteins, unless inactivation of pertinent transcription factors occurred, such as quinone adduction at Cys residues in DNA binding sites, inhibiting transcriptional activation. RTPCR could be employed to determine if mRNA levels of TR and Trx are changed during L-DOPA treatment.

Alternatively, increased degradation could occur as a result of oxidative modifications. Under conditions of oxidative stress with normal initial GSH content, we expect the major oxidative modification to be S-glutathionylation. This modification, normally reversed by Grx, would be sustained due to quinone inactivation of Grx. Increased “irreversible” S-glutathionylation of Trx and TR could signal the protein for degradation. This mechanism could be addressed with chemical inhibition of the proteosome system (i.e. MG132) prohibiting degradation. With proteosome inhibition, if Trx and TR content remained unchanged but activity was lost, an oxidative modification at the active site would be likely. Mass spectral analysis of isolated Trx and TR would determine the nature and location of the modification(s). Such modification would only occur in the cellular milieu, as no diminution of activity is seen when the purified enzymes were challenged with oxidized DOPA (Figure 2-11, p 62). Note, studies examining purified enzyme activity challenged with oxidized L-DOPA were performed in the absence of GSH, and GSH may promote S-glutathionylation of these enzymes at their active site resulting in inactivation. Also, it is possible that S-glutathionylation (or
other oxidative modification) might occur on these enzymes away from their active sites but nevertheless lead to increased degradation and coincident loss of content and activity.

Under conditions of diminished GSH (i.e. BSO treatment) only the activity of TR is lost; content is unaffected. With decreased GSH levels irreversible oxidation, such as sulfonic acid formation, of TR is likely. This alternative oxidative modification may not be sufficient to target TR for degradation. Again, mass spectral analysis of immunopurified TR from SHSY5Y cells with diminished GSH levels would allow characterization of this modification.

**Confirmation of Quinone Adduction to Grx in SHSY5Y Neurons with L-DOPA Treatment:** Grx was shown to be inactivated in vivo in a dose dependent manner (see figure 2.5). Furthermore, Grx was shown to be inactivated in vitro in a time- and dose-dependent manner using isolated Grx and oxidized L-DOPA. These studies were corroborated with mass spectral analysis of purified Grx showing adduction of dopaquinone to the active site Cys 22. Irreversible oxidation of the active site Cys 22 is likely due to its reactive nature at physiological pH with a pKa of 3.5 (J. J. Mieyal et al. 1991a). In order to facilitate detection of the modification in vivo, treatment with radiolabeled L-DOPA would allow the modification to be detected on Western blot (measure radiolabel and co-localize with anti-Grx1 antibody). Alternatively, immunoprecipitation of Grx could be performed along with mass spectral analysis to verify specific adduction of Grx within the cellular context. We expect the results to match the in vitro results.
Oxidative Stress and Modification of Other Proteins Implicated in the Etiology of Parkinson’s Disease: Loss of both Grx and Trx enzymatic systems could result in increased number or extent of cysteine-oxidized proteins. Many proteins are sensitive to sulfhydryl oxidation and have been shown to be important in PD model systems, and mutations in some of these proteins are linked to heritable PD. L-DOPA induced oxidative challenge could alter numerous proteins resulting in a change in normal function and lead to increased cell death. The findings presented in this thesis implicate ASK1 as the major mediator of L-DOPA induced apoptosis. ASK1 activation is initiated by a number of stressors, including endoplasmic reticulum (ER) stress, oxidative stress, UV exposure, calcium signaling and TNFα treatment (J. Matsukawa et al. 2004). Loss of Grx and Trx could lead to ASK1 activation either by direct regulation of ASK1 or indirect regulation of positive and negative regulators of ASK1. Also, activation of ASK1 may occur via upstream mechanisms other than direct regulation of proteins intimately involved in ASK1 regulation (described below).

4.3.1 Regulation of ASK1 Activation:

Direct Modification of ASK1: As with most kinases, ASK1 activity is regulated by its pattern of phosphorylation. Phosphorylation at Ser83 and Ser967 is an inhibitory modification (A. H. Kim et al. 2001; E. H. Goldman et al. 2004). Phosphorylation of Tyr718 targets ASK1 for degradation through its association with SOCS1 (Y. He et al. 2006). Phosphorylation of Thr813, Thr838, and Thr842 are activating modifications leading to downstream phosphorylation of MAPKK (K. Tobiume et al. 2002). Treatment with L-DOPA increases oxidative stress (G. Walkinshaw, C. M. Waters 1995), which
could result in oxidative modification and/or increased degradation of binding partner(s) of ASK1, leading to diminished binding and concomitant activation of ASK1. Alternatively, ASK1 has multiple cysteine residues which also may be oxidatively modified and alter its activity more directly. Previous studies with the LNCaP prostate cell line showed that treatment of ASK1 with N-ethylmaleimide, a thiol modifying agent, activates ASK1. More importantly, menadione (2-methyl-1,4-naphthoquinone) treatment of LNCaP cells resulted in the activation of ASK1 (J. V. Cross, D. J. Templeton 2004). In another study site-directed mutagenesis of Cys residues on ASK1 altered Trx1 binding and phosphorylation of JNK (P. J. Nadeau et al. 2009). Analogous phenomena might occur with L-DOPA treatment, where quinone adduction or other modifications of cysteine residues such as sulfenic acid formation and/or glutathionylation could lead to activation of ASK1. Mass spectral analysis of immunopurified ASK1 from SHSY5Y cells treated with either vehicle or L-DOPA will address direct modification of ASK1 by L-DOPA or its metabolites and should be performed. It is conceivable that such modifications may interfere with phosphorylation of ASK1. In many cases are phosphorylation at specific residues on ASK1 result in inactivation or lead to degradation (A. H. Kim et al. 2001; E. H. Goldman et al. 2004).

Dissociation of Negative Regulators, Grx and Trx, and Activation of ASK1: Both Trx and Grx have been shown to bind ASK1 maintaining its inactive state. However, most studies showing binding to ASK1 have been performed in systems with overexpressed protein. We attempted to immunoprecipitate ASK1 under endogenous conditions and look for Grx and Trx association in both control and L-DOPA treated samples (see
Appendix). However, we were unsuccessful in documenting Grx and/or Trx association in the SHSY5Y neurons, even in the absence of L-DOPA. Possible explanations for the lack of detection of Grx and Trx include limited antibody sensitivity precluding detection of low levels of Grx and Trx, oxidation of components of the complex resulting in dissociation of Grx and Trx from ASK1, or lack of in vivo association of Grx, Trx, and ASK1 in SHSY5Y neurons. In order to address this question further, immunoprecipitation of ASK1 under non-oxidizing conditions could aid in maintaining the complex. However, reducing agents may denature the antibody and thus prevent antibody association to antigen. Crosslinking of the cell lysate could also be performed; however, this prohibited detection of ASK1 by its antibody (See Appendix). Therefore, immunoprecipitation could be attempted in a nitrogen glove box to avoid all contact with oxygen. However, antibody sensitivity would have to be great enough to detect low levels of Grx and Trx since all proteins are not abundant cytosolic proteins. Alternatively, if the complex remains stable throughout the immunoprecipitation process, mass spectral analysis of the ASK1 immunoprecipitated complex could be attempted. Typically the guideline for effective mass spectrometric analysis of proteins is the ability to see Coomassie stained bands for the proteins of interest. Furthermore, to show dissociation of Trx and/or Grx from ASK1 under L-DOPA treatment conditions, the phenomenon would have to be complete dissociation, or quantitative mass spectrometric approaches would have to be employed. These techniques are stretching the current technologies and may not be feasible at present.
Oxidation of Negative and Positive Regulators of ASK1: ASK1 as shown in Scheme 3-1 (Chapter 3, p 92) is negatively regulated by a series of proteins which can be oxidatively modified. ASK1 interacts with numerous proteins including 14-3-3, ASK2, Grx, and Trx among others. Those presented here are negative regulators that have been shown in other contexts to be oxidatively modified resulting in loss of function.

14-3-3 is a multifunctional protein that binds to ASK1, blocking its activation. Furthermore, in a proteomics study 14-3-3 zeta was shown to become oxidized as a result of Aβ treatment of rat primary neuronal cells (R. Sultana et al. 2006). This isoform was also shown to bind and negatively regulate ASK1 (R. R. Subramanian et al. 2004). Future studies should address the potential for oxidation of 14-3-3 in cells under L-DOPA treatment, determining if the oxidation is sufficient to inhibit binding to and negative regulation of ASK1.

Heat shock proteins 72 and 90 (HSP 72, HSP 90 respectively) have been shown to bind ASK1 through different mechanisms. HSP 72 binds ASK1 to prevent homo-oligomerization and activation (H. S. Park et al. 2002), whereas HSP 90 stabilizes the association of Akt and ASK1 resulting in inhibition of ASK1 signaling (R. Zhang et al. 2005). Furthermore, both proteins have been shown to be oxidatively modified with 4-hydroxynoneal treatment inhibiting their ATPase activities (D. L. Carbone et al. 2004; D. L. Carbone et al. 2005). If HSPs are susceptible to oxidative modification, does this inhibit binding to ASK1? Does L-DOPA treatment cause oxidative modification of HSP? What is the nature of the oxidative modification and can it be reversed by either the Trx or Grx systems? Is this reversal impaired with L-DOPA deactivation of the Grx and/or Trx systems?
Another protein associated with both genetic forms of PD is DJ-1, an atypical peroxiredoxin, molecular chaperone, and transcription factor. DJ-1 is mutated with a loss of function or deleted in autosomal recessive PD (P. Heutink 2006) resulting in early onset PD. Furthermore, DJ-1 null mice show increased sensitivity to oxidative stress (R. H. Kim et al. 2005). Recent studies found mutated DJ-1 lymphocytes derived from patients showed increased mitochondrial fragmentation and oxidative stress (I. Irrcher et al. 2010). This new finding shows another potential mechanism by which loss of DJ-1 contributes to oxidative stress. However, it is currently unknown if DJ-1 is oxidized in idiopathic PD and if oxidation of DJ-1 results in loss of protein. However, recently in cell culture models downregulation of Grx was shown to result in oxidation of DJ-1 within the nucleus, and this oxidation is sufficient to allow translocation of Daxx from the nucleus to the cytosol (U. Saeed et al. 2010). This finding presupposes the ability of Grx1 to regulate DJ-1 within the nucleus, or the ability of DJ-1 to be able to translocate freely between nuclear and cytosolic compartments. Daxx, a positive regulator of ASK1, binds and activates ASK1 and its apoptotic pathway (E. Junn et al. 2005). The sensitivity of DJ-1 activity to L-DOPA is unknown, however based on the above results, I hypothesize that L-DOPA treatment may result in oxidation of DJ-1. If DJ-1 can freely move from the nucleus to the cytosol, Grx1 inactivation by L-DOPA or inhibition of the Trx system via loss of protein could allow oxidized DJ-1 to accumulate. Oxidation and thus inhibition of DJ-1 could be the cause of ASK1 activation through its regulation of Daxx. Furthermore, oxidation of DJ-1 is thought to target it for degradation (J. Waak et al. 2009). Other studies have shown oxidized DJ-1 to be a negative regulator of ASK1, binding and inhibiting ASK1 only in its oxidized form (J. Waak et al. 2009). Since these
two results are conflicting, further experimentation would provide insight into the function(s) of DJ-1.

Peroxiredoxin 1 (Prx1), a small non-seleno peroxidase, has been associated with both positive and negative regulation of ASK1. Prx1 functions to reduce peroxides within the cell via a 2-Cys mechanism, and the Prx1 intramolecular disulfide is reduced to its original, active form by the Trx system (M. Aran et al. 2009). Also, Prx-SSG can be reduced by sulfiredoxin (J. W. Park et al. 2009). Several reports have associated Prx1 and ASK1 regulation. In human thyroid cancer cells, inhibition of the proteosome led to apoptosis concomitant with upregulation of Prx1. Knockdown of Prx1 exacerbated the proteosome inhibition and induced death via activation of ASK1 (Z. X. Du et al. 2010). Furthermore, TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) cytotoxicity was increased with knockdown of Prx1 via increased activation of p38 (I. S. Song et al. 2009). MPP+ treatment of PC12 cells led to diminution of Prx1 levels and increased apoptosis (V. T. Chen et al. 2010). Others report binding of Prx1 to ASK1 was increased with hydrogen peroxide treatment, requiring 5mM levels to achieve binding, and ASK1 binding to Prx1 was inhibitory (S. Y. Kim et al. 2008). These two reports provide evidence that Prx1 is a potential negative regulator of ASK1, however they differ in Prx1’s response to oxidative stimulus, MPP+ resulting in dissociation of Prx1 and ASK1 and hydrogen peroxide resulting in association of Prx1 and ASK1. Further studies aimed at evaluating changes in endogenous association of ASK1 and Prx1 under a variety of stress conditions would elucidate the mechanism by which Prx1 appears to negatively regulate the ASK1 pathway, distinguishing whether association or dissociation is stimulus dependent. With L-DOPA treatment and diminution of the Trx system, Prx1
would remain in the oxidized state. Alternatively, L-DOPA treatment may act upon Prx1 in a similar fashion to Trx causing diminution in its content which would also result in decreased association with ASK1 and thus activation of the ASK1 apoptotic pathway.

4.3.2 Upstream Activation of ASK1:

Proteosome Activity and ASK1 Activation: As stated previously, ASK1 can be activated by a number of stressors including proteosome inhibition (J. Matsukawa et al. 2004). Parkin, an E3 ubiquitin ligase, is not only subjected to oxidative stress (K. K. Chung et al. 2004) but has been shown to be mutated with a loss of function in familial PD (M. Westerlund et al. 2010). Furthermore, parkin overexpression has been shown to be protective against proteosome inhibitors, overexpression of α-synuclein, MPTP, and dopamine toxicity (O. A. Levy et al. 2009).

The ubiquitin proteosomal system involves three enzymes which attach a ubiquitin molecule to a protein targeting it for degradation. The first enzyme, the ubiquitin activating enzyme (E1) hydrolyzes ATP and adenylates ubiquitin. This process involves the E1 active site cysteine. The second step is controlled by the ubiquitin conjugating enzyme which acts as a transfer molecule for the ubiquitin. This process also requires an active site cysteine. Finally, the ubiquitin ligase (E3) transfers the ubiquitin from E2 and transfers it to the substrate. Of these three enzymes, the E3 ubiquitin ligase has been shown to be redox sensitive, with an initial increase in activity followed by sustained decrease in activity likely through its self ubiquitination with nitrosylative stress (D. Yao et al. 2004). Also, the 20S component of the proteosome has been reported to be modified oxidatively in yeast treated with hydrogen peroxide resulting in inhibition of function (M. Demasi et al. 2003). Furthermore, inhibition of the proteosome system is
seen in PD models and patients as represented by increased Lewy body formation (K. S. McNaught et al. 2001), and impairment of the proteosome has also been shown to activate ASK1 (J. Matsukawa et al. 2004). As parkin is genetically associated with PD and oxidative modifications result in loss of function, future studies with this protein could provide insight into disease mechanisms and aid in determining a druggable target for PD. In particular, questions regarding parkin and oxidative stress include (1) does inhibition of the proteosome (via lactacystin or MG132) lead to death with similar characteristics to that seen with L-DOPA treatment, (2) does L-DOPA treatment alter the ubiquitin proteosome system via increased oxidation (S-glutathionylation or S-nitrosylation) of E3, (3) does inhibition of the proteosome, either by targeted chemical inhibition or L-DOPA-induced oxidative modification, lead to activation of ASK1 and ultimately phosphorylation of p38 and JNK, and (4) do alterations in either Grx or Trx (via siRNA) lead to inhibition of the proteosome (in the presence and absence of oxidative challenge)? These studies could be addressed in the context of a cellular model similar to the studies presented in this thesis and then translated into a higher animal model system which will be discussed below.

*Calcium Homeostasis and ASK1 Activity:* Calcium dysregulation has also been proposed to be a mechanism of neuronal cell death in PD. Furthermore, model systems involving MPTP show increased cytosolic calcium levels which contribute to overall apoptosis (M. Leist et al. 1998; O. A. Levy et al. 2009). Also, calcium influx into neuronal cells leads to ASK1 activation (J. Matsukawa et al. 2004). Ryanodine receptor 3 is localized to the brain and regulates the release of calcium from the endoplasmic reticulum into the
Previous in vitro studies have shown ryanodine receptor 1 becomes glutathionylated under oxidizing conditions, whereupon this modification causes opening of the receptor and release of calcium into the cytosol (P. Aracena-Parks et al. 2006). To date, no studies have been performed on ryanodine receptor 3 and its potential to be oxidized. Since calcium dysregulation in neurons is intimately associated with death, future studies to examine the role of Grx inactivation and the potential increase in glutathionylation (or other oxidative modification) of the neuronal ryanodine receptor 3 could provide a more complete understanding of mechanisms of alteration of calcium fluxes associated with PD model systems and potentially identify a target(s) to focus therapeutics. Initial experiments measuring cytosolic calcium changes with L-DOPA treatment would aid in validating this hypothesis.

**Protein Aggregation and ASK1 Activity:** Parkinson’s disease patients’ brains show proteinaceous inclusions called Lewy bodies which include α-synuclein and ubiquitin (K. S. McNaught et al. 2001). These inclusions are thought to occur through inhibition of the ubiquitin proteosome system. Proteins are targeted for degradation by ligation of a polyubiquitin chain. Proteins may be targeted for degradation due to misfolding. The unfolded protein response is activated through prolonged stress in the endoplasmic reticulum (ER). ER stress can be induced by numerous mechanisms including increased oxidative stress, increased protein misfolding, increased cytosolic calcium putting the ER potentially at the top of many pro-apoptotic pathways for a variety of insults (I. Kim et al. 2009). Indeed, ER stress due to a variety of stressors, such as paraquat, PolyQ repeats, and TNFα, activates IRE (Inositol-requiring enzyme) which has been shown to lead to
activation of ASK1 through TRAF association. This activation of ASK1 is sufficient to induce apoptosis via phosphorylation of JNK (H. Nishitoh et al. 2002; W. Yang et al. 2009; I. Kim et al. 2009). Knockdown of IRE prior to L-DOPA treatment would test the involvement of this upstream point of initiation of ASK1 activation.

*TNFα and ASK1 Activity:* Another activator of ASK1 is the proinflammatory cytokine, TNFα (Y. Gotoh, J. A. Cooper 1998). Activation is initiated by physical association of adaptor proteins (TNF associated factors) to the TNF receptor (K. P. Hoeflich et al. 1999). TNF associated factor 2 couples the TNF receptor with ASK1 at the N terminus resulting in activation of ASK1. This binding is inhibited by overexpression of Trx as they bind at the same site. This protection from Trx was likely reversed by ROS produced from TNFα treatment (H. Liu et al. 2000). Interestingly, PD patients experience increased inflammation with increased levels of cytokines, including TNFα (discussed below).

### 4.3.3 Inflammation and Parkinson’s Disease

In cell culture models, I found that L-DOPA induces apoptosis via deactivation of Grx and Trx enzymatic systems and concomitant activation of ASK1. However, the brain contains multiple types of cells, not just neurons, and other supporting cells interact with neuronal cells in the brain via cytokine and chemokine release. In order to understand the complexity of PD and contribute to the understanding of current clinical studies examining efficacy of L-DOPA treatment in PD patients, a more complex model system is needed as well as additional studies of the brain environment as a whole.
Mounting evidence implicates inflammation in progression of PD. PD patients exhibit increased cytokines in both peripheral blood mononuclear cells and cerebral spinal fluid. Furthermore, in *post mortem* brain tissue, PD brains showed increased cytokines compared to control (Qian and Flood 2008). Microglial cells are the likely contributors of the excess cytokine production, since these cells are responsible for the innate immunity within the brain. These cells, sequestered in the gray matter, become activated and release cytokines, chemokines, and trophic factors to aid in the immune response (K. G. Fuxe *et al.* 2008). However, the benefits of these cells may be overwhelmed by too much stimulation of inflammatory responses in neurodegenerative disease where a strong correlation exists between PD and inflammation in cell culture, animal models, and patient studies.

Measures of cytokine abundance in peripheral blood mononuclear cells (PBMC) showed PD patients had elevated IL-1B, IL-6, and TNF-α compared to control. Also, isolated PBMC treated with L-DOPA show increased TNF-α production (M. Reale *et al.* 2009). In studies of co-cultures of microglial cells with neurons, lipopolysaccharide (LPS) activates microglia which then kill the neuronal cells, but LPS does not induce cell death in neuronal cultures alone. Also, co-culture of neurons with activated glia (microglia plus astrocytes) leads to greater neuronal sensitivity to both MPP⁺ and 6-hydroxydopamine (D. M. Bronstein *et al.* 1995). Within the central nervous system, TNF-α is considered a major controller of neuroinflammatory events which ultimately lead to neuronal cell death; however in other contexts TNF-α has been implicated in cell survival processes such as differentiation, growth, and proliferation (M. K. McCoy, M. G. Tansey 2008). Samples from PD patients showed elevated TNF-α in cerebrospinal
fluid, brain, and plasma compared to control. Importantly, a mutation in the TNF-α gene elicits increased production of the cytokine and is associated with early onset PD (K. Sriram, J. P. O'Callaghan 2007).

**Grx Regulation of NFκB:** Previous studies in our lab examining the effects of high glucose on retinal glial cells showed increased Grx content and activity as a result of high levels of glucose (M. D. Shelton et al. 2007; M. D. Shelton et al. 2009). This increase in Grx activity resulted in increased NFκB activity and increased cytokine production. By analogy, we hypothesize treatment of microglial cells in culture with TNFα, the major cytokine in PD brain, would lead to increased Grx content and increased NFκB activity. In preliminary studies we find TNFα stimulation of LN18 cells, an immortalized human microglial cell line, leads to increased NFκB activity (Figure 4-1, p 134). In addition, TNFα appears to cause a biphasic alteration in Grx content; at low TNFα concentration Grx appears to be increased, whereas at higher concentrations Grx is diminished (Figure 4-2, p 135). The increase in Grx occurs at a similar concentration of TNFα where the largest increase in NFκB activity has essentially reached a maximum (Figure 4-1, p 134). More studies need to be performed to confirm this relationship. At higher concentrations of TNFα, NFκB levels approach a plateau, and at the highest concentration, the decrease in Grx relative to control is statistically significant (Figure 4-2, p 135). Initial experiments with siRNA knockdown of Grx did not lead to alteration of NFκB activity; however, non-targeting siRNA appeared to lower NFκB activity (Figure 4-3, p 136), making interpretation of these data difficult.
These preliminary experiments were performed in LN18 cells. This cell line was chosen initially to anticipate co-culture of the same species of cells (SHSY5Y and LN18 are both human derived cell lines); however future studies will focus on BV-2 cells, a derived mouse microglial cell line (obtained from Dr. Gary Landreth) because it is considered a better glial cell model for several reasons. Many studies involving use of the BV-2 cells have been reported, providing abundant background information that will be useful. Furthermore, BV-2 cells have been successfully co-cultured with SHSY5Y neurons in multiple instances (proposed co-culture experiments are described below) (H. H. Yu et al. 2008). The key experiment to defeat our working hypothesis would involve transient knockdown of Grx and measurement of NFκB activity in the absence or presence of TNFα. If no changes in NFκB activity are seen, Grx does not regulate NFκB activity in this cell system. However, increased NFκB activity in response to TNFα treatment would be expected to lead to increased cytokine production and be detrimental to surrounding cells in the whole brain, regardless of whether Grx plays a regulatory role. Therefore, co-culture studies with BV-2 and SHSY5Y cells would still provide valuable insights. (see below).

Since inflammation is involved in the neurodegenerative process, the effects of microglial cells co-cultured with SHSY5Y neurons would aid in the understanding of neuronal loss. In preliminary studies, we found that the LN18 cells are resistant to L-DOPA induced apoptosis (Figure 4-4, p 137). Cytokine production was not measured. If cytokine production is enhanced, co-culture studies of BV-2 cells or LN18 cells and SHSY5Y neuronal cells in the presence of L-DOPA may result in enhanced potency of L-DOPA, shifting the dose response curve to the left. These co-culture studies would
**Figure 4-1:** NFκB Is Upregulated with TNFα Treatment of LN18 Cells

Bar graph representing relative luciferase units for LN18 cells transfected with NFκB- and Renilla-reporter gene plasmids. Renilla was used as a measure of transfection efficiency. After transfection with plasmids, cells were treated for 24 hr with TNFα. After harvesting, cells were assayed using the Dual-Luciferase assay system (Promega), measuring emission with a luminometer (SOFTmax PROsoftware, Molecular Devices, Sunnyvale, CA). Triplicate analyses of each of three different preparations (n=3). All TNFα concentrations were significantly different from untreated, p<0.05.
**Figure 4-2: Grx Content Is Differentially Altered by TNFα Treatment**

Bar graph representing Grx content in LN18 cells treated with TNFα for 24 hr. Grx content was obtained by Western blot analysis via densitometry and normalized to the loading control, actin, n≥3. Only 100ng/mL TNFα was significantly different than untreated.
**Figure 4-3:** Knockdown of Grx1 Does Not Alter NFκB Activity in LN18 Cells

(A) Bar graph showing densitometric analysis of knockdown of Grx in LN18 cells. LN18 cells were transfected with siGrx or siSCR3. Western blots were performed and quantitated as described in the Chapter 2. Knockdown of Grx was approximately 50%, n=3 (biological replicates). (B) Bar graph representing NFκB activity. Samples were treated with appropriate siRNA and then transfected with NFκB and Renilla reporter gene plasmids. Luciferase activity was measured from harvested cells using the Dual-Luciferase assay system (Promega), measuring emission with a luminometer (SOFTmax PRO software, Molecular Devices, Sunnyvale, CA). Triplicate analyses of each of two different preparations (n=2).
**Figure 4-4:** L-DOPA does Not Induce Apoptosis in LN18 Cells

Bar graph representing apoptosis as measured by chromatin condensation (Hoechst staining) in LN18 cells treated with L-DOPA for 24 hr. Little, if any, concentration-dependent change in apoptosis was observed compared to untreated samples. Raw values for apoptotic cells are as follows, Untreated: 14.0% ± 1.6%; 50μM L-DOPA: 18.2% ± 1.6%; 100μM L-DOPA: 18.4% ± 3.0%; 250μM L-DOPA: 11.7% ± 1.6%. Triplicate analyses of each of two different preparations (n=2)
provide important insights to guide the design of studies of animals treated with L-DOPA.

4.3.4 Animal Model Studies: Increasing Complication and Physiological Relevance

Advantages and Limitations of Cellular Model Systems: As discussed previously, SHSY5Y dopaminergic neurons are a simple system used to study mechanism of L-DOPA induced cell death (Chapter 1). However, this system is simplified allowing for mechanistic studies to be carried out. Future studies in more complex systems illustrating similar results found here could enhance understanding. Other potential model systems are discussed below.

Cybrids are a hybrid of mitochondria from the platelets of PD patients incorporated into the SHSY5Y transformed cell line. Unlike chemically-induced animal models of PD where MPTP, rotenone, or paraquat typically inhibit Complex I completely, in actual PD patients Complex I activity is not completely lost (S. Gandhi, N. W. Wood 2005). To produce the cybrids, the endogenous mitochondria are disrupted by the selective action of low dose ethidium bromide which has been shown to inhibit mitochondrial DNA and RNA synthesis without inhibiting nuclear DNA and RNA synthesis or cytoplasmic protein synthesis from the recipient cell line (H. B. Horwitz, C. E. Holt 1971). Then the cells without mitochondria are exposed to human platelets, and those that uptake mitochondria are selected because only cells that incorporate mitochondria are able to survive. PD cybrids exhibit decreased Complex I activity, decreased GSH levels, altered calcium homeostasis, and Lewy body formation (P. A. Trimmer et al. 2004). Cybrids are an excellent model to study biochemical pathways,
because these cells are easy to grow and passage, while providing a model system that is more akin to an animal model and contain mitochondria derived from human PD subjects. This model system would provide a second PD model other than ours shown here with diminished GSH due to chemical treatment with BSO. However, pretreatment with ethidium bromide has the potential to alter the original SHSY5Y neuron in other ways making it less like the original cell so additional characterization may be necessary.

Isolated primary cultures of mesencephalic neurons would allow study of key points in a more physiological system. Taken directly from prenatal mice, these cells eliminate the altered characteristics of immortalized cells providing an ex vivo model more similar to actual neurons. Without the ability to continually reproduce, the sensitivity of these cells to L-DOPA may be different from that seen with SHSY5Y cells. I hypothesize that these cells may be more sensitive to drug treatment.

Advantages and Limitations of Animal Model Systems: Ultimately, the findings presented in this thesis warrant further experimentation in a more complex system. An animal model of PD would best examine the effects of L-DOPA in the whole brain. Current animal models have been unable to recapitulate all of the pathological characteristics of PD. Treatment of animals with MPTP, a mitochondrial Complex I inhibitor, provides the model that currently is the most widely accepted toxin model of PD, as chronic treatment with MPTP mimics the majority of PD effects seen in patients. Specifically, MPTP treatment leads to dopaminergic neuronal degradation within the Substantia nigra and striatum, loss of motor function, and increased oxidative stress; however, MPTP treatment does not produce Lewy bodies although other inclusion bodies are seen (M.
Furthermore, treatment with MPTP followed by L-DOPA would provide critical information to further evaluate the potential toxicity of L-DOPA to the whole animal which is compromised in ways that simulate the PD patient. Such studies would provide insights to identify strategies for co-therapy to prevent further loss of dopaminergic neurons. Ideally, measurement of the thiol homeostatic enzymatic activities in dopaminergic neurons and surrounding cells would show if L-DOPA has similar effects as those seen in cell culture. ASK1 has been shown to be activated in previous studies with the MPTP mouse (S. Karunakaran et al. 2007a). Moreover, Grx was shown to be decreased with MPTP (R. S. Kenchappa, V. Ravindranath 2003), but loss of Grx was not examined in the same context as ASK1 activation. These results suggest that the MPTP-treated mouse may be sensitized to L-DOPA toxicity unless there are protective mechanisms in the intact brain that are not evident in the cell models.

A genetic model system would provide benefit over the toxin model system, as PD patients have a slow loss of dopaminergic neurons unlike the quick degeneration in a MPTP model system. Unfortunately, most genetic models formed from known PD mutations have a mild phenotype lacking replication of the disease in humans. The MitoPark mouse involves disruption of mitochondrial transcription via inactivation of the mitochondrial transcription factor A (M. Terzioglu, D. Galter 2008). Since this mutation is under the control of the dopamine transporter, this mutation selectively localizes to dopaminergic neurons and results in decreased mitochondrially produced proteins such as cytochrome C oxidase. These mice display a progressive phenotype similar to PD patients in that they appear normal at birth; however, by 6 weeks loss of dopaminergic neurons is apparent, followed by loss of motor control beginning at 14 weeks of age and
increasing in severity as the mouse ages. Furthermore, these mice respond to L-DOPA treatment and experience the “on-off” phenomenon plaguing many PD patients which requires regimens with increased doses of L-DOPA. However, similar to MPTP treatment, the MitoPark mice do not have α-synuclein positive Lewy bodies; only small cytoplasmic inclusions (M. I. Ekstrand, D. Galter 2009). It would be interesting to treat these mice with L-DOPA to determine if dopaminergic neuronal death is accelerated via ASK1 activation through loss of Grx and/or Trx activity. Measurement of activity of these enzymes specifically would be challenging as the neurons are few in number and difficult to isolate from the adult animals. However, tissue slices may provide answers to increased ASK1 activation and enzyme content through immunostaining. I predict that ASK1 may already be activated in the MitoPark mice, even before L-DOPA treatment due to impairment of mitochondrial respiration which results in increased oxidative stress; and this activation would be increased with L-DOPA treatment due to deactivation of the Grx and Trx systems.

It would be most intriguing to examine the Grx and Trx system and their potential oxidative modification in post mortem brain tissue comparing PD L-DOPA treated tissue to control tissue. Immunohistochemical staining would provide the most straightforward examination of these enzymes co-localizing them with dopaminergic neurons (anti-tyrosine hydroxylase). If antibodies could be made to quinone adducted Grx and oxidatively modified Trx (requiring knowing the most abundant oxidative modification), one could quantitate modified protein compared to total protein to determine if this phenomenon occurs in patients under chronic L-DOPA treatment. Also, ASK1 activation could be measured through either measuring abundance of downstream phosphorylated
targets (*i.e.* phospho-JNK or phospho-p38) or more importantly phospho-ASK1. Because these studies would be performed on *post mortem* brain tissue, care must be taken to ensure that all brain samples were processed in a similar fashion as possible regarding post mortem timing, temperature, etc.
Appendix

A.1 Immunoprecipitation of ASK1:

As mentioned earlier (Chapter 3), ASK1 exists in a signalosome bound to negative regulators when inactive and positive regulators when active (A. Matsuzawa, H. Ichijo 2008). The most direct involvement of Grx1 and Trx1 in regulation of ASK1 activity and associated apoptosis is their binding to ASK1 as negative regulators. Therefore, in order to address if Grx and or Trx act on ASK1 or on upstream activators, I attempted to co-immunoprecipitate Grx1 and/or Trx1 with ASK1 from SHSY5Y cells. I hypothesize that L-DOPA causes dissociation of Grx1 from ASK1 due to dopaquinone adduction at the active site of Grx1. Also, Trx1 dissociation from ASK1 would occur through decreased Trx1 protein content after L-DOPA treatment (as described in Chapter 1). It is the dissociation of negative effectors that results in activation of ASK1, either directly or indirectly through impaired reduction of oxidized modifications on other interacting proteins (See Scheme 3-1, p 92). Multiple co-immunoprecipitation methods were performed to address this hypothesis as detailed below.

Immunoprecipitation Optimization of ASK1: Multiple research groups have demonstrated ASK1 bound to a variety of positive and negative regulators, but in most cases this was accomplished under overexpression conditions of both ASK1 and the “bound” protein of interest. However, Liu et al. reported immunoprecipitation of ASK1 under endogenous conditions in alveolar macrophages. This method was adapted to immunoprecipitate ASK1
from our SHSY5Y cells. The cells were treated with L-DOPA or vehicle and lysed in IP lysis buffer (50mM HEPES pH 7.4, 150mM NaCl, 1% Triton x-100, 0.5% NP40, 1mM sodium orthovanadate, 10mM sodium pyrophosphate, 1mM EGTA, 1mM PMSF, 10% glycerol, 100mM sodium fluoride, 10ng/mL leupeptin, 10ng/mL aprotinin). All protease inhibitors were added fresh. Also, RIPA lysis buffer (1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 150mM NaCl, 10mM EDTA, 50mM Tris pH 7.5) was tested to determine optimal IP conditions. Clarification of lysate through pre-clearing was also tested by incubating lysate in the presence of anti-rabbit IgG and protein A/G beads for 30 min at 4°C. After overnight incubation at 4 °C with 5ng of either anti-ASK1 (Santa Cruz H-300) or anti-rabbit IgG (Santa Cruz J2909) with 1mg of protein lysate, 50μL of protein A/G beads (Santa Cruz SC-2003) were added to the mixture and allowed to rotate for 2-3 hr 4°C. Beads were centrifuged 3000 x g for 3 min at 4°C. Beads were washed four times with ice cold 1xPBS. Protein was eluted from beads by boiling in 2x Sample Buffer with 20 mM DTT for 10 min at 95°C. Prior to loading the SDS-PAGE gel, samples were centrifuged 10,000 x g for 3 min at room temperature. Figure A-1, p 145 is a western blot showing immunoprecipitation efficiency, where IP lysis buffer without pre-clearing was determined to be optimal.

Determination of Binding Partners with ASK1: I hypothesized that loss of Grx activity through dopaquinone adduction (see Section 2.4) and loss of Trx1 content with L-DOPA treatment, would cause activation of ASK1 through dissociation of one or both of these proteins. Both Grx1 and Trx1 require a reduced and functional active site in order to remain bound to ASK1 (M. Saitoh et al. 1998; J. J. Song, Y. J. Lee 2003).
Figure A-1: IP Lysis Buffer Is Optimal for Immunoprecipitation of ASK1 from SHSY5Y Cells

SHSY5Y cells were lysed in either IP lysis buffer (1 and 2) or RIPA buffer (3 and 4). Lysates were pre-cleared with anti-rabbit IgG (1 and 3) and then subjected to immunoprecipitation with overnight incubation of Anti-ASK1 (rabbit) followed by 2 hours incubation with protein A/G beads at 4°C. Lysates in 2 and 4 were not pre-cleared prior to immunoprecipitation. As indicated by the box, ASK1 is ~155kDa. As labeled on the film, “input” shows amount of ASK1 in 100μg of lysate pre-IP. IgG is IP’ed with anti-rabbit IgG; ASK1 is IP’ed with anti-ASK1. “Post” represents supernatant not bound to Ab-protein A/G bead complex. Lysis of SHSY5Y in IP lysis buffer was optimal (lane 2, center of Figure A-1). Pre-clearing the sample was not necessary. The large IgG and small IgG subunits are indicated by the labels (at the right), appearing at 50kDa and 25kDa and accounting for the majority of the bands in the immunoprecipitated samples. Controls were treated with anti- rabbit IgG instead of anti-ASK1 antibody and processed identically to the others.
I first wanted to see if L-DOPA treatment resulted in dissociation of these two proteins. I initially tried to probe for Grx1 and Trx1 after immunoprecipitation of ASK1. However, I was unable to see either Grx1 or Trx1 in the immunoprecipitated samples, despite abundant western blot signals for these proteins in the pre-immunoprecipitated lysate (Figure A-2, p 147).

I hypothesized that the inability to observe co-IP signals for Grx1 or Trx1 for the untreated control could be due to dissociation of the Trx1-ASK1-Grx1 complex while the immunoprecipitation was being performed since it was done under oxidizing conditions, *i.e.* room air, 4°C. Whereas binding of both Grx1 and Trx1 to ASK1 require their active sites to be reduced. A second attempt was made with highly concentrated cell lysate. After lysis, cell lysate was concentrated using Amicon spin concentrators with a molecular weight cut-off of 10,000 Da for 14 min at 14,000 x g 4°C. The resulting concentrated protein was co-incubated with anti-rabbit IgG or anti-ASK1 and prepared in a similar fashion as the above optimized IP conditions. Unfortunately, IP of ASK1 from concentrated samples was not seen potentially due to the inability of the antibody to find the antigen in such a thick protein mixture (Figure A-3 A, p 149).

*Reducing Conditions During IP:* Liu *et al.* used 1mM DTT in the lysis buffer for IP to maintain reducing conditions during IP preserving the ASK1 complex. IP was performed on both regular lysates (data not shown, totally black blot) and concentrated lysates with 1mM DTT (used in Liu *et al.*) or 0.1mM DTT (for concentrated lysates only).
**Figure A-2: Immunoprecipitation of ASK1 Does Not Show Co-**

Immunoprecipitation of Grx and Trx

Immunoprecipitation of ASK1 performed on SHSY5Y cells lysed in IP lysis buffer. A large (16cm x 16cm) gradient gel (8-20%) was run and transferred to PVDF. The lanes of the gel were loaded in the following order (1) pre-IP lysate (input), (2) positive control lysate, (3) 2ng Grx purified protein, (4) 0.5ug Trx purified protein, (5) molecular weight ladder, (6) IgG IP, (7) ASK1 IP, (8) wash #4 IgG, (9) wash #4 ASK1, (10) post IP supernatant IgG, (11) post IP supernatant ASK1. Detection of ASK1 in input, positive control, and ASK1 IP was visible as indicated by boxed area. Trx1 and Grx1 were detected in the input, positive control, purified protein, and post IP supernatant (for Trx1, Grx1 was not seen due to low levels of total protein prohibiting detection by the antibody) indicating the transfer and antibody conditions were successful. No Trx1 was detected with IP. Bands higher than 14kDa in the Trx blot are seen and are potentially small chain IgG. These bands are more visible in the Grx1 blots, where immunoreactivity is seen at a molecular weight higher than the standard and with equal intensity in both IgG and ASK1 IP potentially due to cross reactivity with the anti-rabbit secondary antibody.
Unfortunately, both 1mM DTT as used by Liu et al. and 0.1mM DTT prevented immunoprecipitation of ASK1 (Figure A-3 B, p 149). This is most likely due to the inability of the antibody to bind the antigen as this requires intact disulfide linkages on the antibody. After personal communication with the corresponding authors, it was reiterated the importance of co-immunoprecipitation of ASK1 under reducing conditions and that the immunoprecipitation antibody that was used is no longer available from the company that it was originally purchased from.

Crosslinking of Cellular Proteins to Preserve the ASK1 Complex: Another method to preserve the ASK1 complex is through crosslinking of the sample prior to immunoprecipitation. There are a number of commercially available crosslinkers, most of which rely on disulfide bonding to obtain crosslinking. This is problematic for our samples because both Grx and Trx bind ASK1 through their active site which contains Cys residues which must be reduced presumably to form an intermolecular disulfide bond with ASK1.

Formaldehyde is a small crosslinking reagent that is reversed with heat treatment (C. Klockenbusch, J Kast 2010). Pretreatment of SHSY5Y cell pellets with formaldehyde was as follows, 5 minutes of 1% or 0.5% formaldehyde in 1xPBS, room temperature, followed by 3 min centrifugation of 3000 x g. Once pelleted, supernatant was immediately removed and replaced with 1.25M glycine in 1xPBS to quench the formaldehyde and prevent further crosslinking and centrifuged 3 min at 3000 x g. This was repeated one time prior to lysis in IP lysis buffer.
Figure A-3: Neither Concentration of SHSY5Y Lysates Nor Inclusion of Reducing Agent Preserved Signalosome Complex

(A) Concentration of SHSY5Y lysates was done by centrifugation with Amicon 10MWKO concentrators. After concentration, BCA protein analysis was performed to determine protein needed for IP. Small reacti-vials with a mini magnetic stir rod were used to circulate the antibody/lysate mixture. Protein A/G beads were applied for 3 hours at 4°C after overnight incubation of lysate and antibody. No immunoreactivity for ASK1 was seen after IP. I hypothesize that too much protein was used in a concentrated amount prohibiting the antibody from interacting with the antigen. (B): Samples were processed similar to 3A with the exception that 0.1mM DTT or 1mM DTT were included in the lysis buffer. DTT prohibited any IP, as seen previously in non-concentrated samples (data not shown).
BCA protein analysis was performed, and it was noted that the total protein was much less than expected (1.5mg versus 5 mg seen without crosslinking prior to lysis for equal amounts of SHSY5Y cells). Samples were run on a SDS-PAGE gel with and without heat treatment to reverse crosslinking. After transfer to PVDF membrane, blots were probed as usual with anti-ASK1 (Figure A-4 A, p 151). Unfortunately, crosslinking inhibited the ability to detect ASK1 on the western blots. I hypothesize that crosslinking may block antibody binding sites on ASK1, as interpreted for certain sites on integrin beta1 after formaldehyde crosslinking (C. Klockenbusch, J Kast 2010). Diminution of the amount of formaldehyde did not aid in the ability to detect ASK1 by western blot (Figure A-4 B, p 151).

A second commercially available crosslinker was also tried. Disuccinimidyld suberate (DSS) is a membrane permeable crosslinker that contains amine reactive esters at each end. This commonly used crosslinker allows for stabilization of protein protein interactions. 5mM DSS was added to PBS and incubated with the cell pellet for 30 min at room temperature. The crosslinking was quenched with addition of Tris buffer pH7.5 to a final concentration of 20mM. Immunoprecipitation proceeded as described above. Total protein was diminished in crosslinked samples as seen with formaldehyde treatment. Figure A-5, p 152 shows a western blot of samples after immunoprecipitation. ASK1 was not detectable in crosslinked samples.
**Figure A-4: Crosslinking with Formaldehyde Precludes Detection of ASK1 via Western Blot**

(A) Crosslinking with 1% formaldehyde was performed prior to lysis of SHSY5Y cells with IP lysis buffer. Other lanes include 100ug and 200ug of lysate from SHSY5Y cells lysed in IP lysis buffer. Crosslinking of SHSY5Y prohibited detection by anti-ASK1 (lane 1). (B): crosslinking with 1% formaldehyde followed by quenching with 1.25M glycine (1 and 2) or with 0.5% formaldehyde followed by quenching with 1.25 M glycine (3 and 4). Lanes 1 and 3 were subjected to boiling for 10min at 95°C, lanes 2 and 4 were not boiled. No reactivity is seen with anti-ASK1.
Figure A-5: Crosslinking with DSS Prohibits Detection of ASK1 Following Immunoprecipitation

Crosslinking with 5mM DSS was performed prior to lysis of SHSY5Y cells with IP lysis buffer. Lanes labeled left to right include 2ng Grx, 0.5μg Trx, IP with anti-IgG antibody, IP with anti-ASK1 antibody, molecular weight ladder, lysate used in IP, and supernatants removed after immunoprecipitation. Proteins could not be detected after crosslinking with DSS. The top portion of the gel was probed with anti-ASK1 antibody (labeled ASK1); bottom portion of the gel was probed with anti-Trx antibody (labeled Trx).


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