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Department of the Army position, policy or decision unless so
designated by other documentation.
The overall goal of the proposal was to provide a detailed assessment of the role of oxidative damage in Parkinson's Disease (PD) postmortem brain tissue, body fluids of PD patients and in the MPTP model. There were trends towards increased oxidative damage markers in post mortem brain tissue of PD patients and a number of specific genes linked to oxidative stress were reduced in expression. There was increased lipid peroxidation in progressive supranuclear palsy brains. There were no significant alterations in 8-hydroxy-2-deoxyguanosine in the plasma of PD patients. We found that overexpression of manganese superoxide dismutase attenuated MPTP toxicity whereas its deficiency exacerbated toxicity. Similarly, a deficiency of glutathione peroxidase exacerbated MPTP neurotoxicity. Overexpression of Bcl2 or a dominant-negative mutant of interleukin-1 converting enzymes significantly attenuated MPTP toxicity. We found increases in oxidative damage markers in the substantia nigra of MPTP treated baboons. A number of free radical spintraps as well as a selective inhibitor of neuronal nitric oxide synthase protected against MPTP neurotoxicity. In summary, our studies showed further evidence linking oxidative damage to dopaminergic neurons in the substantia nigra as well as in the MPTP model of PD.
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4. **TABLE OF CONTENTS**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Page #</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Front Cover</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>SF298</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Foreword</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Table of Contents</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>Body</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>Key Research Accomplishments</td>
<td>14</td>
</tr>
<tr>
<td>8</td>
<td>Reportable Outcomes</td>
<td>16</td>
</tr>
<tr>
<td>9</td>
<td>Conclusions</td>
<td>18</td>
</tr>
<tr>
<td>10</td>
<td>References</td>
<td>20</td>
</tr>
<tr>
<td>11</td>
<td>Appendices</td>
<td>21</td>
</tr>
<tr>
<td>12</td>
<td>Personnel</td>
<td>21</td>
</tr>
</tbody>
</table>
5. INTRODUCTION

The overall goal of the original proposal was to provide a detailed assessment of the role of oxidative damage in Parkinson's Disease. There were three specific aims. The first was to examine oxidative damage in postmortem brain tissue of patients with Parkinson's Disease and parkinsonian disorders. The second was to develop novel HPLC based assays for quantification of oxidative damage in body fluids of Parkinson's Disease patients and to attempt to develop new biomarkers. The third specific aim was to determine whether oxidative stress plays a key role in neuronal death, which occurs in MPTP-induced Parkinson's Disease. During the course of the grant, we made considerable progress in achieving these aims. Following initiation of the program, we unfortunately suffered the loss of one of the co-investigators, Dr. John B. Penney, Jr., who died unexpectedly on January 31st of 1999. His position was then filled by Dr. Anne B. Young, Chief of Neurology at Massachusetts General Hospital.
6. BODY

We initially carried out studies of postmortem brain tissue in patients with progressive supranuclear palsy which is a parkinsonian syndrome. We examined a well-established marker of oxidative damage to lipids in the subthalamic nucleus and cerebellum from 22 patients with progressive supranuclear palsy and 11 age-matched controls using sensitive HPLC techniques. In progressive supranuclear palsy, we found a significant increase in tissue malondialdehyde levels in subthalamic nucleus as compared to the age-matched control groups.

We also carried out studies of oxidative damage to lipids in the frontal cortex of 14 pathologically confirmed cases of PSP and 13 age-matched controls. We found significant decrease in $\alpha$-ketoglutarate dehydrogenase complex, which is known to be sensitive to oxidative damage. There are also significant increases in tissue malondialdehyde levels in the superior frontal cortex of PSP samples.

We completed a comprehensive study of the activity and localization of two essential antioxidant systems (superoxide dismutase enzymes and total glutathione) in seven brain regions of postmortem control and parkinsonian PSP brains. The most robust finding of this study was a striking increase in SOD1 activity and glutathione levels within most of the PSP brain regions examined relative to controls. The subthalamic nucleus showed a significant increase in SOD2.

We have conducted studies in which we have directly examined alterations in gene expression in dopamine neurons in human PD. It is important to recognize that the pathology of PD is complex. Not only is there a very substantial loss of dopaminergic neurons, but there is also an accompanying proliferation of glial cells. Thus, in a midbrain of advanced PD, the majority of the cells present will not be dopaminergic neurons, and many will be non-neuronal cell types. In this circumstance, a simple regional approach to gene expression, such as studying homogenates of the midbrain, will likely produce a mixed result reflecting both loss of dopaminergic neurons and increased expression of inflammatory and glial genes. In order to examine directly the regulation of gene expression in dopaminergic neurons in PD, we have employed the recently developed technology of laser capture microdissection [Emmert-Buck et al., 1996].

We have used an Arturus PixCell II, an instrument developed for studies of cancer biology, but recently applied to the central nervous system. This instrument uses an ethylene vinyl acetate film which is applied to thin tissue sections, and an infrared laser which melts the film overlying cells of interest, allowing their selective removal from the section. Using protocols which we have developed in our laboratory, we have been able to extract mRNA from small numbers (100-500) of neurons, amplify the mRNA using a T7-polymerase based method, and perform both real-time PCR analysis of mRNA abundance as well as expression array analysis.
We have used LCM to collect and analyze melanized dopamine neurons from the substantia nigra of PD and normal control brains. A total of eight cases have been examined so far, four PD and four controls, with comparable ages and post-mortem intervals. Prior to preparing sections for LCM, we evaluated each case for integrity of RNA. This was accomplished by extraction of RNA from a small block of tissue, and analysis using an Agilent BioAnalyzer Biosizing Analysis. This provides the equivalent of an electrophoretic analysis of RNA, but can be performed rapidly and on small quantities of tissue. Only cases exhibiting good preservation of RNA, as indicated by the presence of sharp 18 and 28S ribosomal subunit bands, were used for LCM. From each of the eight cases, we collected 500 neurons from the SNpc, and performed two rounds of T7 polymerase aRNA amplification. Biosizing Analysis was used to confirm the products of amplification (Figure 1). The amplified RNA was used to prepare cDNA, and this was subsequently labeled for hybridization to expression arrays. In these studies we have used the Research Genetics “Named Human Genes” (GF211) array, which contains cDNAs for 4325 distinct genes. Hybridized arrays were visualized using a Packard Optiquant phosphoimager, and the arrays analyzed using Research Genetics Pathways 4 software.

![Figure 1. Agilent Biosizing analysis of mRNA amplified from human control (left) and PD (right) SN neurons](image)
Overall the patterns of hybridization obtained from the eight different pools of human SNpc dopamine neurons were remarkably similar. (Figure 2). A parallel analysis of gene expression in a different region of human brain, the caudate, revealed a substantially different pattern of expression, demonstrating that the expression patterns were specific to SNpc dopaminergic neurons.

Figure 2. Array from control and PD dopamine neurons. In top panels densities indicate mean values for 4 control and 4 PD cases. Values are plotted below.
A group comparison between the mean hybridization intensities in the control and PD groups revealed that the great majority of genes were expressed at similar levels in the two groups (Figure 2). Using a t-statistic to screen for significant differences, we found differential expression of a total of 53 genes (Figure 3). Nineteen of these were more abundant in the controls, while 34 were more abundant in PD. A correlation between the intensity of the hybridization signals and the direction of change was apparent, with downregulation of highly expressed genes in PD.

**Table 1: Genes differentially expressed in PD vs control dopamine neurons.**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
<th>Control</th>
<th>PD</th>
<th>Ratio (PD/Control)</th>
<th>Difference (PD-Control)</th>
<th>p-value</th>
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<tr>
<td>A3D4</td>
<td>Actinin, alpha 3</td>
<td>0.057</td>
<td>0.028</td>
<td>2.078</td>
<td>0.029</td>
<td>0.017</td>
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<tr>
<td>A415415</td>
<td>Amyloid beta (A4) precursor</td>
<td>0.056</td>
<td>0.039</td>
<td>1.425</td>
<td>0.017</td>
<td>0.017</td>
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<tr>
<td>A45349</td>
<td>Vav 2 oncogene</td>
<td>0.059</td>
<td>0.038</td>
<td>1.553</td>
<td>0.021</td>
<td>0.016</td>
</tr>
<tr>
<td>A447279</td>
<td>Transducin-related enhancer of solit 2, homolog of Drosoohiia E(so1)</td>
<td>0.057</td>
<td>0.036</td>
<td>1.614</td>
<td>0.021</td>
<td>0.016</td>
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<tr>
<td>A54130</td>
<td>TFAP2B</td>
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<td>0.036</td>
<td>1.638</td>
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<td>0.019</td>
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<td>A61580</td>
<td>MAD1 (mitotic arrest deficient, yeast, homoloo)-like 1</td>
<td>0.057</td>
<td>0.036</td>
<td>1.608</td>
<td>0.022</td>
<td>0.018</td>
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<td>A62046</td>
<td>PTEN</td>
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<td>1.618</td>
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<td>G00908</td>
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<td>0.036</td>
<td>1.608</td>
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<td>G00910</td>
<td>MAD1 (mitotic arrest deficient, yeast, homoloo)-like 1</td>
<td>0.057</td>
<td>0.036</td>
<td>1.608</td>
<td>0.022</td>
<td>0.018</td>
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**Figure 3.** Genes differentially expressed in PD vs control dopamine neurons.

Principal Investigator: M. Flint Beal, M.D.
Some of the genes revealed by this approach to be differentially regulated have previously been implicated in the pathobiology of PD, while others are novel. Interestingly, 3 potentially protective enzymes (paraoxonase 3, superoxide dismutase 1, and gamma-glutamyltransferase) were among the downregulated genes, whereas Hsp40 (HDJ1) and TNF were upregulated. Our results show that paraoxonase 3 mRNA is reduced by almost 30% in remaining neurons in PD substantia nigra pars compacta. Paraoxonase 3 is an arylesterase that hydrolyzes organophosphates such as pesticides, with activity similar to paraoxonase 1. Recent studies have shown that paraoxonases play a major role in the detoxication of organophosphate (OP) compounds processed through the P450/PON1 pathway. Because of the significance of organophosphates in idiopathic Parkinson disease a number of reports have tried (not always successfully) to link particular paraoxonases genotypes to PD [Akhmedova et al., 2001; Carmine et al., 2002; Taylor et al., 2000].

Another factor that has been previously linked to PD is glutathione. Several studies show decreased levels of glutathione (GSH) in PD and other oxidative stress-related neurodegenerative diseases [Perry et al., 1982; Riederer et al., 1989]. Our preliminary data shows that γ-glutamyltransferase (also called γ-glutamyltranspeptidase) is downregulated in PD. This enzyme is responsible for the conversion of GSH to glutamate. A by-product of this reaction is cysteinylglycine which is the recycled in the GSH synthesis loop by two enzymes, a dipeptidase and glutathione synthetase. The reduction in the mRNA for this enzyme that we observe may be a reflection in the reduction of the GSH itself or it could be responsible for a reduced GSH synthesis due to a reduction in the substrate for the GSH synthetase.

Superoxide dismutase 1 (SOD1) has been shown to be protective of neurons exposed to 6-hydroxydopamine in animal models of PD. In our study mRNA for SOD1 was significantly decreased in PD nigra dopaminergic neurons. Interestingly, studies of blood antioxidant profiles of living PD patients show increased SOD1 activity [Illic et al., 1999; Serra et al., 2001]. In a previous study of PSP, a parkinsonian disorder, we observed a similar phenomenon: SOD1 activity was increased in the brain overall, but this was the result of both a decrease in neuronal SOD and an increase in glial SOD [Cantuti-Castelvetri et al., 2002].

Our data also shows upregulation of Hsp40 (HDJ1). This chaperone colocalizes to α-synuclein containing inclusions in LB neuropathological tissue [McLean et al., 2002] and is upregulated in DLB. Even more striking, in a screen in drosophila of more than 7000 P-element insertions, the homolog of Hsp40 was one of only 2 proteins identified which suppress polyglutamine toxicity (the other was also a chaperone, dTRP2) [Kazemi-Esfarjani et al., 2000].
We measured 8-hydroxy-2-deoxyguanosine and 3-nitrotyrosine concentrations in the substantia nigra of PD brains. There was great variability, which precluded our ability to observe any significant changes. More recently, we obtained further parkinsonian tissue from the German Brain Bank and examined matrix metalloproteases in this tissue. Matrix metalloproteases are known to be induced by oxidative damage such as that produced by nitric oxide, reactive oxygen species and metabolites of the arachadonic pathway. In the postmortem brain tissue of 10 PD cases as compared to 10 control cases, there were no alterations in MMP-9 expression. There were reduced MMP-2 levels in PD cases as compared to age-matched controls and increases in TIMP-1, the endogenous tissue inhibitor of MMPs. Taken together, these alterations of MMPs and TIMPs suggest that MMP/TIMP dysregulation may play a role in the pathogenesis of PD.

The second objective was to develop novel HPLC based assays for quantification of products of oxidative damage in human plasma samples, and to determine whether these biomarkers are altered in inpatients with Parkinson’s Disease. We developed a novel column-switching assay to measure 8-hydroxy-2-deoxyguanosine concentrations in a large number of biological matrices. This was an HPLC electrochemical column-switching technique based on a unique selectivity of carbon columns that enables them to selectively concentrate 8-hydroxy-2-deoxyguanosine. We also established an assay for nitro-gamma-tocopherol as well as coenzyme Q10 for measurements in plasma.

These studies were completed. We examined 25 patients with idiopathic Parkinson’s Disease meeting well-defined clinical criteria and 20 control subjects. The concentrations of OH^dG were 26.1 +/- 3.1 ng/ml in PD subjects and 25.4 +/- 2.4 ng/ml in control subjects. In addition, we carried out studies of coenzyme Q10 levels and nitro-gamma-tocopherol in patients who participated in a clinical trial examining the effects of coenzyme Q10 administration as a neuroprotective agent in PD. We found that oral supplementation with coenzyme Q10, produced dose-dependent increases in coenzyme Q10 levels from a mean of 0.82 +/- .24 to 1.78 +/- .19 at a dose of 300 mg daily to 2.12 +/- .13 at 600 mg per day and 3.94 +/- .24 at 1200 mg per day. The baseline values were not significantly different from those seen in controls. There were no alterations in gamma-nitro-tocopherol values in the PD patients, as compared to controls at baseline or following coenzyme Q10 supplementation.

A major part of our proposal was to examine oxidative damage and whether it played a role in the pathogenesis of the MPTP model of Parkinson’s Disease. We carried out a number of studies, which examined MPTP neurotoxicity in transgenic mice as well as the effects of a number of therapeutic interventions. We initially studied mice that overexpress manganese superoxide dismutase by approximately 50% in brain homogenates. We found that MPTP toxicity was significantly attenuated in these mice. A significant increase in 3-nitrotyrosine levels was seen in littermate controls that was not observed in the transgenic mice overexpressing manganese SOD. We examined
MPTP toxicity in mice which overexpressed the antiapoptotic protein bcl2. Bcl2 is one of the primary proteins, which inhibits apoptotic cell death and it has also been demonstrated to exert antioxidant effects in vitro. We found that bcl2 overexpressing mice had significant reductions of dopaminergic neurotoxicity following administration of MPTP on either an acute or chronic dosing regimen. Bcl2 also blocked MPP+ induced activation of caspases and MPTP induced increases in 3-nitrotyrosine levels.

We also examined the effects of MPTP in mice with a dominant-negative mutant interleukin-1 converting enzyme. We showed that these mice are highly resistant to MPTP toxicity. There was also protection against MPTP induced depletion of tyrosine hydroxylase immunoreactive neurons.

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We examined whether there was evidence of increased oxidative damage in the substantia nigra of baboons following administration of MPTP. We found that there was a significant increase in 3-nitrotyrosine immunostaining of substantia nigra dopaminergic neurons that was markedly attenuated by administration of a neuronal nitric oxide synthase inhibitor. We also found that there was an increase in α-synuclein staining in the substantia nigra neurons of these primates. This was the first evidence to show a direct linkage between oxidative damage and deposition of α-synuclein a major component of Lewy bodies found in PD.

We examined whether a number of novel free radical spintraps can inhibit MPTP neurotoxicity. Treatment with cyclic nitrone free radical spintrap MDL 101-002 significantly attenuated MPTP dopaminergic neurotoxicity as well as increases in 3-nitrotyrosine concentrations. We also examined the effects of a novel neuronal nitric oxide synthase inhibitor, AR17338. This nNOS inhibitor exhibited dose-dependent protection against MPTP induced dopaminergic neurotoxicity. We also examined the effects of creatine on MPTP neurotoxicity. We found that oral supplementation with creatine, which can buffer against ATP depletion produce significant protection against MPTP induced dopamine depletions in mice and also protect against loss of nissl and tyrosine hydroxylase stained neurons.

We carried out studies of MPTP toxicity in mice which have a deficiency in cellular glutathione peroxidase. We found that glutathione peroxidase knock-out mice show significant increases and hydroxyl radical generation following administration of the mitochondrial toxin, malonate. Administration of MPTP resulted in significantly greater depletions of dopamine, DOPAC and HVA in the glutathione peroxidase knock-out mice as compared to those seen in wild-type control mice. We also examined the effects of MPTP in mice, which have a 50% deficiency in manganese superoxide dismutase, which is the major free radical scavenging enzyme within mitochondria. These mice showed a significant increase in vulnerability to dopamine depletion following administration of MPTP. There was also a significantly greater reduction in dopaminergic neurons in the substantia nigra of these mice.
In summary, our studies have provided further evidence for oxidative damage as playing a critical role in PD pathogenesis. We found evidence of increased oxidative damage in postmortem brain tissue of both PD subjects as well as from patients suffering from the related disorder PSP. We did not find increased OHβdG or nitro-gamma-tocopherol levels in plasma of PD patients suggesting that the oxidative damage in the brain is not reflected in plasma, or that its contribution to over all oxidative damage markers in plasma is insufficient to significantly alter them. We carried out a large number of studies of various transgenic mouse models which have alteration in free radical scavenging enzyme or in antiapoptotic proteins. These studies consistently show that increases in free radical scavenging enzymes were protective whereas reductions exacerbated MPTP neurotoxicity.
7. KEY RESEARCH ACCOMPLISHMENTS

1. The finding of increased oxidative damage to lipids in the subthalamic nucleus in cerebellum from 22 patients with progressive supranuclear palsy as compared to 11 age-matched controls. This was a significant increase in tissue malondialdehyde levels as measured by a specific and sensitive HPLC procedure.

2. The finding of increased oxidative damage to lipids in the frontal cortex of 14 pathologically confirmed patients with progressive supranuclear palsy as compared to 13 age-matched controls.

3. The finding of a significant decrease in $\alpha$–ketoglutarate dehydrogenase complex in the frontal cortex of patients with progressive supranuclear palsy.

4. The finding of a robust increase in SOD-1 activity and glutathione levels within most of the PSP brain regions as compared to controls.

5. The finding of a significant increase in manganese superoxide dismutase activity in the subthalamic nucleus of PSP patients.

6. The development of methods to laser capture individual dopaminergic neurons in PD. This has then been utilized to extract mRNA from small numbers (100-500 neurons) and to the amplify the mRNA using real time PCR analysis of mRNA abundance as well as expression array analysis.

7. A detailed analysis of gene expression patterns in substantia nigra dopaminergic neurons in PD as compared to controls. A differential expression of 53 genes was observed.

8. A finding that several genes involved in metabolism or oxidative stress responses are altered in PD substantia nigra pars compacta. Paraoxonase-3 was down regulated, as was superoxide dismutase 1, and gammaglutamyl transferase whereas HSP40 and TNF were upregulated.

9. The finding of trends towards increased 8-hydroxy-2-deoxyguanosine and 3-nitrotyrosine concentrations in substantia nigra PD brains, however, we were unable to find significant alterations.

10. The finding of reduced activity of MMP-2 in PD cases as compared to age-matched controls and increases in TIMP-1, the endogenous tissue inhibitor of MMPs. These findings may be a consequence of oxidative stress.

11. The finding that there are no alterations in concentrations of 8-hydroxy-2-deoxyguanosine in plasma of PD subjects as compared to normal controls.

12. The findings of dose-dependent increases in coenzyme Q10 following oral administration, yet no alterations in gamma-nitrotocopherol levels.

13. The finding that mice, which overexpress manganese superoxide dismutase show significant attenuation of MPTP neurotoxicity. Increases in 3-nitrotyrosine seen in littermate controls were not observed in mice overexpressing manganese SOD.

14. The finding that mice that overexpress the antiapoptotic protein Bcl2, which also reduces oxidative stress, are resistant to either acute or chronic administration of MPTP.

15. The finding that mice with a dominant-negative mutant of interleukin-1 converting enzyme are resistant to MPTP toxicity and show sparing of tyrosine hydroxylase immunoreactive neurons.

16. The finding that MPTP administration to baboons results in increased 3-nitrotyrosine immunostaining of substantia nigra dopaminergic neurons consistent with involvement of peroxynitrite.
17. The finding that following administration of MPTP to baboons there is an increase in α-synuclein staining in the substantia nigra pars compacta.

18. The finding that novel free radical spintraps can inhibit MPTP neurotoxicity. Specifically, we found that the spintrap MDL101-002 significantly attenuated MPTP neurotoxicity as well as increases in 3-nitrotyrosine concentrations.

19. The finding that a novel highly specific neuronal nitric oxide synthase inhibitor dose-dependently protected against MPTP induced neurotoxicity.

20. The finding that oral administration of creatine and cyclocreatine produce significant protection against MPTP neurotoxicity.

21. The finding that MPTP toxicity is exacerbated in mice with a knockout of glutathione peroxidase. We found that these mice showed increases in hydroxyl radical generation in significantly greater depletions of dopamine and tyrosine hydroxylase neurons following administration of MPTP.

22. The finding that mice, which have a 50% deficiency in manganese superoxide dismutase, show increased vulnerability to both dopamine depletions as well as loss of tyrosine hydroxylase neurons following administration of MPTP.
8. REPORTABLE OUTCOMES


Principal Investigator: M. Flint Beal, M.D.


9. CONCLUSIONS

We carried out a large number of studies, which directly addressed the role of oxidative damage in PD as well as other parkinsonian disorders. We found trends towards increases in 3-nitrotyrosine and 8-hydroxy-2-deoxyguanosine in PD substantia nigra however there was too much variability for these changes to become significant. We did establish increased lipid peroxidation in the parkinsonian syndrome progressive supranuclear palsy. We were able to demonstrate increased malondialdehyde concentrations in the subthalamic nucleus, frontal cortex and cerebellum. We also found increased immunostaining for SOD1 and SOD2 in the subthalamic nucleus. There is also an increase in glutathione levels, which may be compensatory for oxidative stress. We directly examined gene-altered expression in dopaminergic neurons of human PD. We found that there were reductions in 3 potentially protective enzymes. These are paraoxonase-3, superoxide dismutase 1 and gamma-glutamyl transferase. Gamma-glutamyl transferase is responsible for the conversion of glutathione to glutamate. A by-product this reaction cystinylglycine which is then recycled in glutathione synthesis loop. A reduction in the messenger RNA for this enzyme may therefore be due to a reduction in glutathione itself. Previous studies have documented reductions in glutathione and the substantia nigra of PD patients. It is plausible that this could contribute to increased vulnerability to oxidative stress.

We measured 8-hydroxy-2-deoxyguanosine concentrations in plasma of PD patients using a highly specific and novel column switching technique with electrochemical detection. We found no significant alterations in the PD plasma, suggesting that oxidative damage is not reflected in plasma of PD patients. We observed alterations in matrix metalloproteases in the substantia nigra of postmortem tissue suggesting that alterations and dysregulation of matrix metalloproteases may play a role in PD pathogenesis.

We carried out extensive studies documenting the important role of oxidative stress in the MPTP model of PD. We demonstrated that a number of alterations in antioxidant enzymes significantly either attenuate or exacerbate MPTP toxicity. Specifically we found that overexpression of manganese superoxide dismutase attenuated MPTP toxicity whereas its deficiency exacerbated the toxicity. Similarly a deficiency of glutathione peroxidase exacerbated MPTP neurotoxicity. We found that overexpression of Bcl2 or of a dominant negative mutant of interleukin-1 converting enzyme both significantly attenuated MPTP neurotoxicity. We demonstrated that there are increases in oxidative damage markers in the substantia nigra of MPTP treated baboons as well as increases in α-synuclein staining. Lastly we found that a number of novel free radical spin traps as well as a selective inhibitor of neuronal nitric oxide synthase protect against MPTP neurotoxicity.
In summary, our studies have provided further evidence for oxidative damage both in PD post mortem substantia nigra as well as in the related parkinsonian syndrome progressive super nuclear palsy. We did not find alterations in plasma of PD patients when we measured 8-hydroxy-2-deoxyguanosine concentrations. We documented a large number of studies, which clearly implicate oxidative stress in the pathogenesis of MPTP neurotoxicity.
Principal Investigator: M. Flint Beal, M.D.

10. REFERENCES


11. APPENDICES

12. PERSONNEL

David Albers
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