

## Protein Nitration in Parkinson's Disease

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**Abstract.** Oxidative stress has been proposed as a pathogenetic mechanism in Parkinson's disease (PD). One mechanism of oxidative cellular injury is the nitration of protein tyrosine residues, mediated by peroxynitrite, a reaction product of nitric oxide and superoxide radicals. We demonstrate here the presence of nitrotyrosine immunoreactivity in Lewy bodies within melanized neurons and in amorphous deposits associated with intact and degenerating neurons. The core of the Lewy body was frequently intensely immunolabeled, while the rim was lightly labeled or unlabeled. This likely reflects the fact that tyrosine residues of neurofilament proteins are primarily localized to Lewy body cores, and suggests that nitrotyrosine is present in neurofilament protein itself. Although these observations are as yet unable to provide a definitive link between oxidative stress and neuronal dysfunction, they demonstrate that oxidative stress has occurred within the vulnerable neurons of PD, leaving a permanent marker of oxidative modification of neuronal proteins within the target cells of neurodegeneration. In addition, these observations provide a potential link between excitotoxicity and oxidative stress within the vulnerable neurons of PD and represent a pathogenetic mechanism in common with the 2 other major age-related neurodegenerative diseases, Alzheimer disease and amyotrophic lateral sclerosis.

**Key Words:** Excitotoxicity; Lewy body; Nitrotyrosine; Oxidative stress; Parkinson's disease.

### INTRODUCTION

Oxidative stress has been proposed as a pathogenetic mechanism for the 3 major age-related neurodegenerative diseases: Alzheimer disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (1). The most compelling evidence for oxidative stress and damage has been developed in PD, where a decrease in substantia nigra glutathione content (2), lipid peroxidation (3), DNA hydroxylation (4), elevation of intraneuronal iron (5, 6), and damage to complex I of the mitochondrial electron transport chain (7) have consistently been found. The presence of such cellular injury implies that oxidative stress plays a role in the pathogenesis of Parkinson's disease.

The results derived from such tissue analyses have demonstrated regional oxidative injury, but have not further defined alterations of the substantia nigra in specific cellular populations. A more detailed analysis of pathological changes in defined cellular populations can be accomplished with antibodies that recognize specific oxidative modifications. Protein nitration is one such oxidative modification that can be demonstrated at the cellular level in neurodegenerative diseases, indicating that a sequence of specific oxidative reactions has taken place. Nitration of tyrosine residues has been hypothesized to represent a major mechanism of oxidative modification of proteins, producing dysfunctional proteins (8)

and killing cerebellar neurons in culture (9). Nitrotyrosine formation has been demonstrated in atherosclerotic lesions (10) and has also been proposed for a role in neurodegenerative disease (11–15).

Using this approach, we have recently described the presence of nitrated tyrosine residues colocalized to neurofibrillary tangles in hippocampal specimens from patients with AD (13). In the present study, we have employed an antinitrotyrosine antibody to demonstrate the presence of aberrantly nitrated proteins in the substantia nigra pars compacta (SNc) of Parkinson's disease patients.

### MATERIALS AND METHODS

Five Parkinson's disease patients and 4 controls were used in the present study. All specimens were derived from the Mount Sinai Alzheimer's Disease Research Center brain bank or the Mount Sinai Hospital autopsy service. Clinical and pathological data relating to these cases is given in the Table. The Parkinson's disease cases had typical histories of chronic progressive parkinsonism, and neuropathological analysis demonstrated prominent Lewy body formation and loss of pigmented neurons in the substantia nigra pars compacta. Control cases had no clinical features of neurological disease and on neuropathological examination were free of significant abnormalities. All brain specimens were fixed in 10% neutral buffered formalin for 2 to 4 weeks, blocked, and embedded in paraffin. Sections from the midbrain were cut at 5  $\mu$ m from paraffin-embedded blocks, deparaffinized, and hydrated through graded alcohols. Sections were then pretreated for one hour with 0.1% aqueous saponin (Sigma Chemical, St. Louis, Mo), microwaved in 0.1M aqueous sodium citrate pH 6.5, and finally treated with 3% aqueous hydrogen peroxide to remove endogenous peroxidase activity. Immunocytochemistry was performed using a rabbit polyclonal primary antibody (Upstate Biologicals, Lake Placid, NY) diluted 1:400 in 0.01M phosphate buffered normal saline (PBS) with 1% normal goat serum at room temperature overnight. Sections were further processed by the avidin-biotin

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Supported by AG02219 and AG14382 from the National Institutes of Health and the Lowenstein Foundation.

method (Vector, Burlingame, Calif) and immunoreactivity was visualized with diaminobenzidine. Finally, sections were briefly counterstained with cresyl violet for Nissl substance. Control specimens were prepared by preincubation of antisera with 1 mM nitrotyrosine (Aldrich Chemical, Milwaukee, Wis) before overnight incubation of sections and processing as for experimental sections.

## RESULTS

Within the SNc of Parkinson's disease patients, nitrotyrosine immunolabeling was associated with the cell bodies of melanized neurons and appeared in differing morphological configurations. In many instances, there were irregular accumulations of intracellular immunoreactivity intermingled with neuromelanin or in isolated clusters (Fig. 1A, C). Nitrotyrosine immunoreactivity was also closely associated with the remnants of degenerated dopaminergic neurons, displaying little but incontinent neuromelanin and remaining nitrotyrosine immunoreactivity (Fig. 1B). Other immunolabeled structures had the clear morphological appearance of Lewy bodies (Fig. 1A, D) or appeared to be irregular smaller Lewy bodies (Fig. 1B). Such immunolabeled structures were round, occurred either singly or in multiples, and were confined to intact somata within melanized neurons. This characteristic Lewy body appearance was further highlighted by differential immunoreactivity, such that the core and ring were easily discernible, the core being intensely labeled and the annular rim lightly labeled or unlabeled.

Midbrain specimens from neurologically normal cases demonstrated no discernible nitrotyrosine immunoreactivity within a normal density of melanized neurons (Fig. 1E). Preincubation controls were also negative for nitrotyrosine immunoreactivity.

## DISCUSSION

We have demonstrated the presence of nitrotyrosine immunolabeling in the SNc of Parkinson's disease patients in contrast to its absence in control specimens. The presence of oxidized lipids (3), DNA (4), and proteins (present study) in PD indicates that oxidative attack on biological macromolecules may underlie the tissue damage seen in this disease. The present results lend further evidence to the concept that oxidative stress plays a role in PD and demonstrate that oxidative injury has occurred within the vulnerable nigrostriatal dopaminergic neurons of PD and has left a permanent marker of the oxidative modification of neuronal proteins.

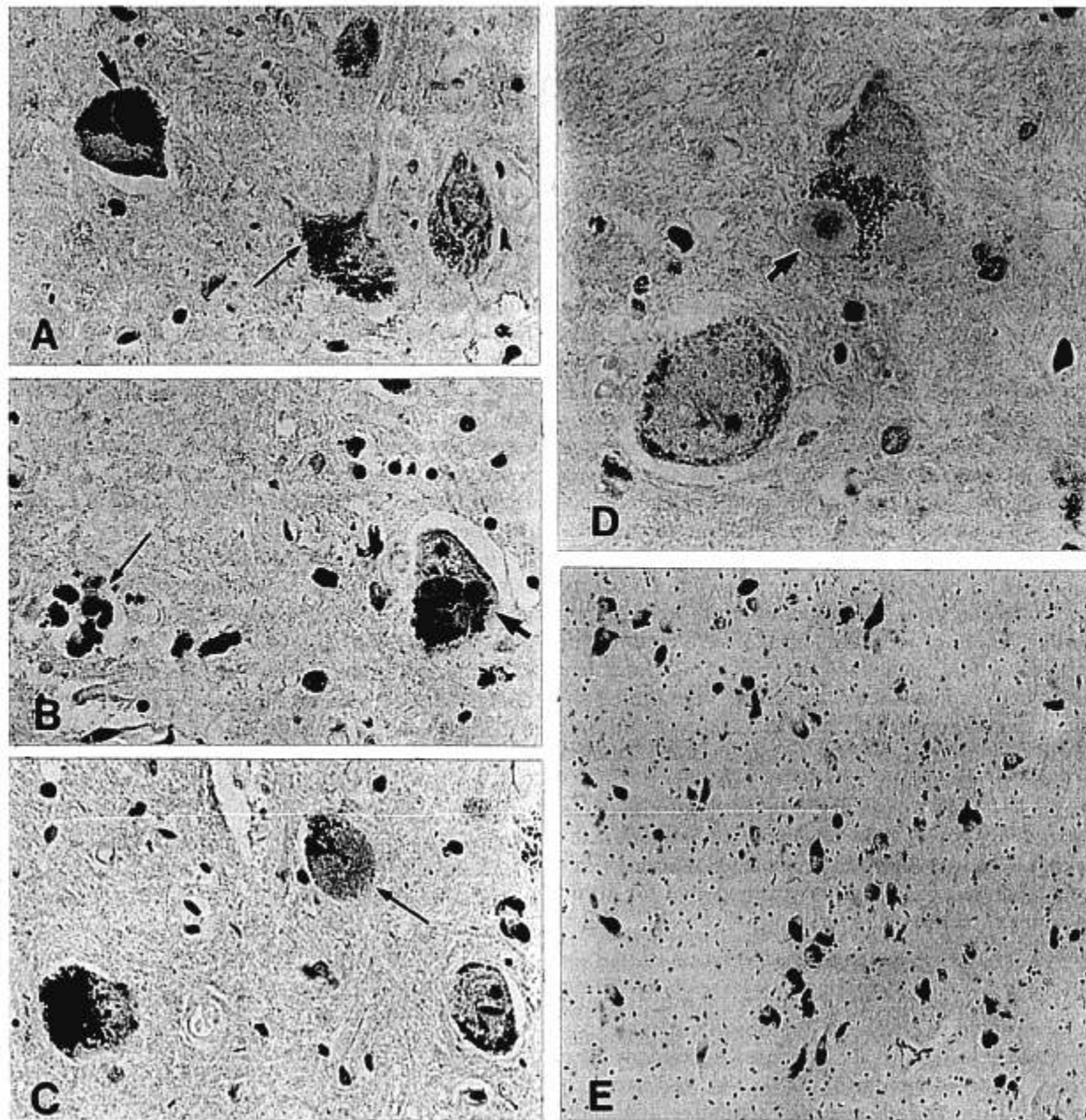
Although such oxidative damage is likely to be detrimental, the precise relationship between oxidative stress and cellular alterations in PD is uncertain. Protein nitration presents one specific scenario, relating excitotoxicity and oxidative stress to the production of dystrophic intraneuronal inclusions. The stimulation of NMDA receptors, known to be present on nigral dopaminergic neurons, will lead to increased free intracellular calcium and

the production of both nitric oxide, mediated by the activation of neuronal nitric oxide synthase (16), and superoxide via the arachidonic acid cascade (17). Nitric oxide and superoxide react at a near diffusion-limited rate to produce peroxynitrite, which will attack the phenol ring of tyrosine residues when catalyzed by iron or superoxide dismutase (18). It is significant in this context that we (6) and others (5) have shown increased iron localized to melanized neurons of the substantia nigra in Parkinson's disease. NMDA receptor stimulation may occur either by glutamatergic activity, or by relief of the NMDA receptor's magnesium block secondary to mitochondrial dysfunction postulated by the "weak excitotoxic hypothesis" (19). In the present study the demonstration of nitrated tyrosine localized to degenerating neurons in the SNc of Parkinson's disease patients indicates that peroxynitrite mediated protein nitration is a likely mechanism for the production of degenerative changes in this disorder.

Hantraye et al (20) have provided further support for the relevance of peroxynitrite-mediated neurodegeneration to Parkinson's disease. Their study demonstrated that inhibition of neuronal nitric oxide synthase by 7-nitroindazole prevented neuronal damage in the substantia nigra, and alleviated parkinsonian movements in MPTP intoxicated baboons. Their findings further suggest that peroxynitrite-mediated neuronal injury subsequent to the production of superoxide and nitric oxide plays a role in the neuronal damage seen in PD.

There are 2 potential implications of the morphological characteristics of the antinitrotyrosine immunolabeling we have observed in Parkinson's disease. First, the amorphous character of some deposits of immunoreactivity in the absence of clearly defined Lewy bodies, both within intact neurons and among the debris of degenerated neurons, indicates that protein nitration may occur either prior to, or independently of, Lewy body formation. The irregular nitrotyrosine-immunoreactive accumulations in many instances appeared to form spherical aggregations, but whether these structures are pre-Lewy bodies cannot be determined.

Second, the distinct pattern of antinitrotyrosine immunolabeling of Lewy bodies lends itself to a clearer interpretation. Studies have demonstrated that Lewy bodies are largely composed of neurofilament protein (21, 22). Extensive epitope mapping by Schmidt and coworkers (23) demonstrated a differential distribution of neurofilament epitopes within the Lewy body. In their study, immunolabeling of Lewy bodies by a large panel of monoclonal antibodies demonstrated that the core of the Lewy body is composed almost exclusively of the rod portion of neurofilament molecules, while the multiphosphorylation sites found at the tail are concentrated in the peripheral rim of the Lewy body. Tyrosine residues are



**Fig. 1.** Antinitrotyrosine immunolabeling of midbrain sections of Parkinson's disease and control specimens. A–D, midbrain specimens from Parkinson's disease cases; E, control specimen. Immunolabeled Lewy bodies (short arrows A, D) within pigmented neurons. Note intense immunolabeling of the Lewy body core and light labeling of the rim. Within some of the pigmented neurons were amorphous (A, thin arrow, C) or Lewy body-like (B, short arrow) areas of immunoreactivity, while other immunolabeled material was associated with the remnants of degenerated neurons (B, thin arrow). Neurologically normal control cases (E) showed no antinitrotyrosine immunoreactivity. Original magnifications: A–C, 100 $\times$ ; D, 150 $\times$ ; E, 25 $\times$ .

concentrated almost entirely in the rod portion of neurofilament subunits, suggesting that antinitrotyrosine antisera are in fact labeling nitrated tyrosine residues of the neurofilament rod domain integral to Lewy bodies.

These observations connect the key intracellular marker of Parkinson's disease, the Lewy body, with evidence that oxidative stress has occurred within the vulnerable neurons of PD. Substantia nigra pars compacta neurons express both

NMDA receptors as well as neuronal nitric oxide synthase (16). The capacity to generate nitric oxide intraneuronally, combined with superoxide production from dopaminergic metabolism, can render SNc neurons acutely vulnerable to nitrotyrosine-mediated neurodegeneration. Our demonstration of the presence of nitrotyrosine immunoreactivity within neuronal Lewy bodies lends evidence to the notion that this sequence of events has in fact occurred.

TABLE

	Age	Duration	CDR	Tangles	Cause of death	PMI
PD1	81	4 years	0.5	None	Ischemic heart disease	2 h 40 m
PD2	77	2 years	1	Moderate	Bronchopneumonia	2 h 20 m
PD3	85	1 month	2	Moderate	Acute subdural hemorrhage	5 h 15 m
PD4*	81	2 years	n.a.	Sparse	n.a.	n.a.
PD5	91	5 years	4	Sparse	Ischemic heart disease/ bronchopneumonia	3 h 10 m
C1	42				Ischemic heart disease	10 h 40 m
C2	70				Metastatic melanoma	7 h
C3	57				Cardiorespiratory arrest	n.a.
C4	79		1		Ischemic heart disease/ Lymphoma (ileum)	21 h 40 min

Duration indicates the time from clinical diagnosis of Parkinson's disease to death. CDR is the Clinical Dementia Rating of the PD cases, if known. Tangles indicates the extent of neurofibrillary tangle formation in the hippocampus and parahippocampal gyrus. None of the PD cases demonstrated neocortical tangles. PMI is the postmortem interval from death to autopsy. The clinical records of the control cases were reviewed and there were no indications of underlying neurological disease other than C4, who had a history of depression. h = hours; m = minutes. \* Only specimens comprising neocortex, hippocampus/parahippocampal gyrus and midbrain were available from this case for confirmation of the diagnosis of Parkinson's disease.

The 3 major neurodegenerative diseases of aging demonstrate the intracellular deposition of altered cytoskeletal proteins. Neuronal cytoskeletal proteins provide stabilization of the intricate dendritic and axonal processes necessary for neurotransmission. Pathological alteration of these structural proteins or other critical biological molecules such as occurs in the nitration of tyrosine residues can be expected to have major deleterious effects on neuronal function. The presence of nitrotyrosine residues within both Lewy bodies as we have demonstrated here, and within neurofibrillary tangles of Alzheimer disease (13) as we have previously demonstrated, provides further evidence for a common link between the degenerative processes of these age-related neurodegenerative diseases.

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Received October 28, 1997

Revision received January 9, 1998

Accepted January 9, 1998