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BRIEF COMMUNICATION

Overexpression of Neutrophil Neuronal Nitric Oxide Synthase in Parkinson's Disease

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Much evidence supports a role of nitric oxide (·NO) and peroxynitrite ($ONOO^-$) in experimental and idiopathic Parkinson's disease (PD); moreover, an overexpression of neuronal nitric oxide synthase (nNOS) was recently reported in the basal ganglia of PD patients. In accord, we previously found a 50% increased ·NO production rate during the respiratory burst of circulating neutrophils (PMN) from PD patients. As PMN express the nNOS isoform, the objective of the present study was to ascertain whether this increased ·NO production is representative of nNOS gene upregulation. PMN were isolated from blood samples obtained from seven PD patients and seven age- and sex-matched healthy donors; nNOS mRNA was amplified by reverse transcriptase–polymerase chain reaction and the products were hybridized with a probe for nNOS. Nitrotyrosine-containing proteins and nNOS were detected by Western blot and NO production rate was measured spectrophotometrically by the conversion of oxymyoglobin to metmyoglobin. The results showed that both ·NO production and protein tyrosine nitration were significantly increased in PMN isolated from PD patients (PD 0.09 ± 0.01 vs 0.06 ± 0.008 nmol min^{-1} 10^6 $cells^{-1}$; $P < 0.05$). In addition, five of the seven PD patients showed about 10-fold nNOS mRNA overexpression; while two of the seven PD patients showed an expression level similar to that of the controls; detection of nNOS protein was more evident in the former group. In summary, it is likely that overexpression of nNOS and formation of $ONOO^-$ in PMN cells from PD patients emphasizes a potential causal role of ·NO in the physiopathology of the illness.

Key Words: **nitric oxide; Parkinson's disease; neutrophils; peroxynitrite; neuronal NOS; neurodegeneration.**

Parkinson's disease (PD)[2] is a neurodegenerative disorder characterized by a selective loss of dopaminergic neurons in the tier ventral of the pars compacta of the substantia nigra (SNpc) (1).

Although the etiology of PD remains unknown, genetic and environmental factors, acting either alone or combined, have been implicated (2). Most published evidence suggests that nigral apoptotic death seems to depend on the development of oxidative stress (3–6). However, possible pathogenic effects of nitric oxide (·NO) and of the product of its

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[2] Abbreviations used: PD, Parkinson's disease; nNOS, neuronal nitric oxide synthase; PMN, NO production rate during the respiratory burst of circulating neutrophils; SNpc, pars compacta of the substantia nigra; RT-PCR, reverse transcriptase–polymerase chain reaction.

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reaction with superoxide anion, the strong oxidant peroxynitrite ($ONOO^-$), have recently been hypothesized as well (6). In accordance, high levels of 3-nitrotyrosine, a "footprint" of previous exposure to $ONOO^-$, has been found in the central core of Lewy bodies, the pathological hallmark of PD (7). In addition, these observations are in line with the recent description of Eve *et al.*, who reported nNOS overexpression in the brains of patients with Parkinson's disease (8).

The selective damage of neuromelanized dopaminergic neurons in the SNpc and the representative biochemical neuronal changes in PD are extended to extraneural tissues such as platelets and lymphocytes (9). Circulating neutrophils are suitable to study the role of ·NO in PD at the peripheral level since they express nNOS (10) and simultaneously release ·NO, $O_2^{\cdot-}/H_2O_2$, and $ONOO^-$ when exposed to appropriate stimuli (11). Moreover, we detected about 60% increased ·NO production rate of activated neutrophils from PD patients when compared with healthy controls (12). In the same way, Barthwal *et al.* measured an increased nitrite content in neutrophils, but not in platelets, of PD patients, which suggested a disregulation of nNOS gene rather than eNOS gene, coding the isoform currently expressed in platelets (13). On this basis, the aim of our study was to explore whether, in PD, the expression of nNOS is increased in circulating neutrophils, as a model of nNOS-containing cells outside the central nervous system.

PATIENTS AND METHODS

Patients. This study included the collection of venous blood samples from seven PD patients, both newly diagnosed and chronically treated with L-dopa only (mean daily dose 542 ± 191 mg), and from seven age- and sex-matched healthy controls. All patients met United Kingdom Parkinson's Disease Brain Bank clinical diagnostic criteria for idiopathic PD (14). Controls were subjected to clinical and neurological examination and did not have signs of extrapyramidal disorders or a familial history of Parkinson's disease. The following exclusion criteria were applied to both groups: intake of antioxidants, L-dopa agonists, MAO-B inhibitors, or aspirin and the presence or previous history of severe systemic disease and dementia. All treated patients were allowed to stop L-dopa administration the day before at 10 PM and venous samples were invariably taken at 9 AM.

Neutrophil isolation. Neutrophils were isolated by dextran sedimentation followed by Ficoll-Hypaque centrifugation at room temperature as previously described (15). The preparation was >98% neutrophils and the viability >96% as assessed by the trypan blue exclusion test.

Nitric oxide and hydrogen peroxide production. ·NO production was measured by the oxidation of oxymyoglobin to metmyoglobin and hydrogen peroxide by the *p*-hydroxyphenylacetic acid/horseradish peroxidase assay, before and after addition of 0.1 μg/ml PMA (15).

Reverse transcriptase–polymerase chain reaction (RT-PCR). Purified PMN cells, as well as skeletal muscle obtained during surgical procedures, were homogenized in guanidine thiocyanate buffer to extract total RNA as described (16), and cDNAs for nNOS and β-actin were synthesized using human nNOS-specific reverse primer or poly-T. Aliquots of cDNA were subjected to PCR (MJ Research, Inc., Watertown, MA) using specific pairs of primers for human nNOS (forward: 5′-CCGGAGACATCATTCTTGCGG-3′, reverse: 5′-TCCTGCCCATCATCGTAGGC-3′) and for β-actin. PCR consisted of 15 cycles (for β-actin) and 30 cycles (for nNOS) of denaturation (94°C, 1 min), annealing (64°C, 1 min), and extension (72°C, 1 min) followed by a final 5-min extension step.

Southern blot. Total amplified products were loaded in 2% agarose gels and visualized by ethidium bromide staining of the gels. Southern blot analysis and hybridization at 42°C for 1 h with [γ-^{32}P]ATP 5′-end-labeled oligonucleotide probe (5′-CACGTGGTCCTCATTCTG-3′) were performed. Autoradiography was allowed for 16 h at room temperature, and signals were quantified with a Shimadzu chromatoscanner (Tokyo, Japan). Values were individually normalized to the corresponding level of β-actin.

Western blotting. Pellets containing 10^7 cells were lysed in 100 μl of 20 mM Hepes, 1 mM dithiothreitol, 2 μg/ml leupeptin, 10 μg/ml pepstatin, 50 μM phenylmethylsulfonyl fluoride, 50 mITU aproti-

TABLE I

Clinical Features of Patients with Parkinson's Disease and Controls

	Parkinson's disease	Controls
Age (years)	64 ± 2.5	62 ± 4
L-dopa treated:untreated ((*n*))	5:2	–
Men:women ((*n*))	4:3	4:3
Hoehn and Yard stage	II–III	–
Duration of disease (years)	7.9 ± 2.6 (1–20)	–

nin, and 0.8% deoxycholic acid, pH 7.2, kept on ice for 1 h, sonicated at 60 W for 30 s (Cole–Parmer Instruments, Vernon Hills, IL), and centrifuged at 10,000*g* for 10 min. Fifty micrograms of protein was subjected to SDS–PAGE and transferred to a nitrocellulose membrane. After the membranes were washed and blocked, the first antibody at 1:1000 dilution was applied for 1 h, washed, subjected to a second goat anti-rabbit IgG antibody conjugated to alkaline phosphatase at 1:3000 dilution for another hour, and developed with a chemiluminescent system.

Antibodies and reagents. Polyclonal anti-human nNOS was purchased from Upstate Biotech (Lake Placid, NY) and polyclonal rabbit anti-nitrotyrosine antibody was a generous gift from Drs. Alvaro G. Estévez and Joseph S. Beckman (University of Alabama at Birmingham). Goat anti-rabbit IgG conjugated to alkaline phosphatase and the chemiluminescent detection kit were from Bio-Rad (Hercules, CA). Acrylamide and sodium dodecyl sulfate were purchased from GIBCO BRL (Gaithersburg, MD). Tetramethylammonium chloride was from Fisher Scientific (Fair Lawn, NJ), *Taq* DNA polymerase and the kit for cDNA synthesis were from Promega (Madison, WI), and Hybond-N^+ nylon membranes were from Amersham (Buckinghamshire, UK). All other reagents were from Sigma–Aldrich Co. (St. Louis, MO).

Statistical analysis. Statistical differences were assessed by the unpaired Student's *t* test.

RESULTS

The clinical characteristics of the patients and age- and sex-matched control subjects are summarized in Table I. In accordance with our previous findings (12), in the PD group, the PMA-stimulated ·NO production rate was increased by 60% ($P < 0.001$). The H_2O_2 production rate during the respiratory burst was not different between the groups.

The nNOS mRNA was detected by RT-PCR in all neutrophil samples; the expression level of nNOS was increased in five of seven of the PD samples, while two were similar to the respective controls. Therefore, parallel experiences in the control samples required an increase in the cell number for detection. In this setting, densitometric measurements normalized to β-actin content were consistent with an average 10-fold nNOS mRNA overexpression in the neutrophils isolated from PD patients (Fig. 1a).

In accordance with the increase in nNOS mRNA, the immunodetection of nNOS protein was confirmed by the presence of a single band of 160 kDa in four of five of the PD samples, identical to that present in rat cerebellum homogenates and not detectable in control samples (Fig. 1b). The results seem to be important considering that (a) nNOS protein of normal human neutrophils is at the threshold level of this technique (10) and (b) nNOS protein was particularly evident in those PD samples with the highest mRNA levels (Table II). In this way, the finding of clearly detectable nNOS protein

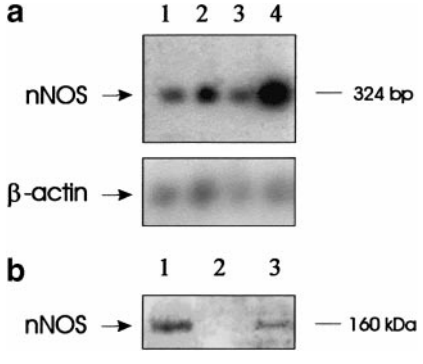

FIG. 1. Neuronal NOS overexpression in neutrophils from PD patients. (a) Total neutrophil RNA from control (1 and 3) and PD (2 and 4) individuals was analyzed by RT-PCR, Southern blot, and hybridization with [γ-^{32}P]ATP 5′-end-labeled nNOS (upper panel) and β-actin (lower panel) probes. (b) Western blot of 50 μg of total soluble and weakly membrane bound proteins of neutrophils from control (lane 2) or PD subjects (lane 3). A band of 160 kDa identical to the positive control obtained from rat cerebellum lysate (lane 1) is revealed with anti-human nNOS antibody (Upstate Biotech, Lake Placid, NY) at 1:1000 dilution.

TABLE II

Nitric Oxide Synthase Activity and Expression in Neutrophils of Patients with Parkinson's Disease

	Patient						
	1	2	3	4	5	6	7
NO production rate[a] (nmol min^{-1} 10^6 $cells^{-1}$)	0.95 (0.54)	0.89 (0.45)	0.76 (0.50)	0.67 (0.42)	0.7 (0.49)	0.93 (0.61)	0.52 (0.44)
nNOS mRNA	+	++	++++	+++	++++	+	++
nNOS protein	+	ND[b]	++	++	+++	ND	–

[a] NO production rate of controls in parentheses; mean ± SE: PD patients 0.77 ± 0.06 vs controls 0.49 ± 0.03 ($P < 0.001$).
[b] ND, not done.

in human brain is associated with 20-fold more nNOS mRNA than in neutrophils (10).

In PD patients, the overexpression of nNOS was even associated with a 50% increase in the basal ·NO production rate ($P < 0.05$; Fig. 2a). This result should explain the finding of nitrotyrosine-containing proteins in resting nonstimulated neutrophils from the patients with the highest cell nNOS levels (Fig. 2b) and likely indicates that the cells were significantly exposed to peroxynitrite in terms of concentration × time.

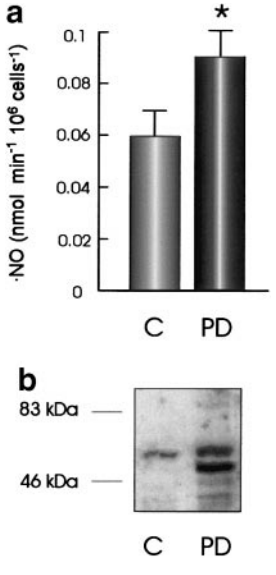

FIG. 2. ·NO production (a) and nitrotyrosine detection (b) in resting neutrophils isolated from patients with Parkinson's disease (PD) and healthy controls (C). In (a), mean values ± SE differed significantly as assessed by the unpaired Student's *t* test (*$p < 0.05$, $n = 7$). (b) Representative Western blot of 50 μg of total soluble and weakly membrane bound proteins of the neutrophils as performed with a 1:1000 anti-nitrotyrosine antibody dilution.

DISCUSSION

The results of the present study are consistent with overexpression of nNOS in circulating neutrophils of PD patients. These preliminary results extend the postmortem studies of Eve *et al.*, who described similar findings in selected regions of the subthalamic nucleus and globus pallidus (8). Both increased ·NO production rate and the presence of tyrosine-nitrated proteins in neutrophils from PD patients reflect the nNOS overexpression as well. Furthermore, they are consistent with the finding of 3-nitrotyrosine staining in the SNpc of PD patients (7). Certainly, tyrosine nitration is directly related to the cell exposure to $ONOO^-$ (17), and its formation rate *in vivo* is often dependent on ·NO concentration, the limiting species in neutrophils (18).

The modulation of nNOS expression in circulating neutrophils of PD patients could be related to a number of transcription factors reported to participate in its brain regulation, such as Oct-2, which stimulates nNOS gene transcription (19), or to mutational events that strengthen the promoter activity, possibly due to the influence of specific environmental agents related to PD prevalence (2). Interestingly, ·NO synthesis seems to be tightly regulated since an average 10-fold nNOS overexpression produced an increase of only 60% in NOS activity. In this regard, no correlation was observed between the expression and activity of NOS. This discrepancy could be related to several factors; for example, ·NO itself binds to the heme prosthetic group of NOS, exerting a negative feedback mechanism (20).

Although peripheral or extraneural findings might partially reflect the events occurring in the

central nervous system, we can hypothesize that nNOS overexpression in neutrophils could be related to a common disorder in nNOS isoforms. In this context, it is known that tissue-specific regulation of the nNOS gene includes different posttranslational modifications related to the subcellular location and to the phosphorylation state. However, a correspondence between changes in neutrophil and brain nNOS subtype is emphasized by the fact that, in this study, the RT-PCR strategy was designed to amplify a common region of all reported nNOS alternative spliced products. Moreover, though there are no data in human neutrophils, in addition to the whole messenger, there exist two alternative spliced forms of human nNOS mRNA associated with the deletion of exons 9 and 9–10. However, these variants represent less than 5% of the total messenger, they are thought to be inactive, and their relative content was similar in all studied tissues (brain, kidney, and skeletal muscle) (21). The presence of circulating factors in the plasma of parkinsonian patients able to stimulate the production of NO by neutrophils of healthy subjects (22) could be involved in the upregulation of nNOS. On the other hand, previous studies proposed that overexpression of nNOS could be an adaptive mechanism secondary to decreased dopaminergic activity (8). Nevertheless, the finding of nNOS overexpression in cells not exposed to a high dopamine turnover does not support the latter hypothesis.

At present, it is not defined whether nNOS activation of $nNOS^+$ cells is the cornerstone for the events ending in a selective dopaminergic damage. Nevertheless, a generalized increase in ·NO production and $ONOO^-$ formation could likely contribute to excytotoxicity, mitochondrial damage (23), and neuron apoptosis in Parkinson's disease.

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