Mol Neurobiol (2016) 53:3309–3314
DOI 10.1007/s12035-015-9274-3

Association of Polymorphism of Neuronal Nitric Oxide Synthase Gene with Risk to Parkinson's Disease

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Received: 17 March 2015 / Accepted: 28 May 2015 / Published online: 17 June 2015

Abstract Environmental factors are implicated in aging as well as genetic predisposition-induced Parkinson's disease (PD) pathogenesis. Wrongdoers increase oxidative stress and nitrosative burden, which eventually degenerate the nigrostriatal dopaminergic neurons. Inhibition of the expression of nitric oxide synthase (NOS), an enzyme responsible for nitric oxide (NO) biosynthesis, prevents the demise of the nigrostriatal dopaminergic neurons. Polymorphism of NOS is thus expected to alter PD susceptibility. The study therefore aimed to examine an association of neuronal NOS (nNOS) gene polymorphism with nitrite, an indicator of nitrosative load; lipid peroxidation, an index of oxidative stress and PD susceptibility. An age-matched case-control study was performed in the north Indian residents enrolled at the Neurology Department of the King George's Medical University, Lucknow, India. While nNOS exon 29 TT variant genotype [odds ratio (OR)=2.20, 95 % CI=1.08–5.34, P=0.040], combined TT and CT variants [OR=1.68, 95 % CI=1.05–2.69, P=0.031] and T allele [OR=1.58, 95 % CI=1.10–2.28, P=0.014] were found to be significantly associated with PD susceptibility, no association between nNOS exon 18 [OR for TT carriers=1.97, 95 % CI=0.89–4.20, P=0.09 and OR for T allele=1.35, 95 % CI=0.94–1.93, P=0.098] and PD risk was observed. Lipid peroxidation was augmented in all patients irrespective of their genotype. While genotype independent increase in nitrite content was observed in PD patients of exon 29 polymorphic groups, only heterozygous variant genotype of exon 18 was associated with augmentation in nitrite level as compared with respective control. The results obtained thus demonstrate that selected nNOS polymorphisms do not significantly contribute to PD risk in north Indian population.

Keywords Parkinson's disease · Neuronal nitric oxide synthase · Polymorphism · Nitrite · Lipid peroxidation

Introduction

Parkinson's disease (PD) is a sporadic and chronic movement disorder, associated with the progressive demise of the nigral dopamine-producing neurons [1–3]. Despite imprecise etiology and diagnosis, it is clinically distinguished from other neurodegenerative disorders due to the presence of a few motor and non-motor anomalies [4–6]. Reduced oxidative and nitrosative burden partially rescue from PD and toxins-induced Parkinsonism [7]. While such aspects incredibly help in explaining PD pathogenesis, entire mechanism remains to be elusive [8]. Nitric oxide (NO) is recognized as the main troublemaker that increases intracellular nitrosative stress. Nitric oxide synthases (NOSs), which catalyze NO formation in the neurons, are classified in two main categories. The first is called neuronal NOS (nNOS, NOS1) while the second is referred to as inducible NOS (iNOS, NOS2) [7, 9]. Expression of iNOS is regulated by many external and internal factors while the expression of the former remains to be constitutive.

Exonic single nucleotide polymorphism (SNP) alters the structure and function of a gene while intronic SNP alters

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the activity and SNP located in un-translated region changes the stability of the messenger ribonucleic acid [7]. Since NO production depends on nNOS activity, alteration in its expression and/or catalytic activity could amend the level of nitrosative stress in dopaminergic neurons. Alteration in the sequence of iNOS is important predominantly when environmental conditions are favorable for PD onset. Therefore, it was worthwhile to investigate the association of polymorphism of nNOS gene with PD risk and also with oxidative stress and nitrosative load that contribute to PD pathogenesis. Although nNOS interacts with environmental risk factors associated with PD [10, 11], conflicting results deny this notion. A few SNPs in nNOS are found to be associated with PD risk in a population while the same SNPs are not found to be associated in other population [10, 12–15]. Although lack of association in a few studies [13, 16] is reported, it is widely accepted that nNOS polymorphism acts as modifiable factor of PD risk. Owing to variation in environment and lifestyle factors as well as ethnicity of north Indians as compared with the rest of the world, it was worthwhile to investigate the association of NOS1 SNPs with PD risk. Since two SNPs located in exon 18 and exon 29 of nNOS gene exhibit variable association with PD risk in different populations [10, 12–17], the current study aimed to investigate their association with PD risk and also with nitrosative and oxidative stress indicators.

Materials and Methods

Materials

Restriction enzymes-DraIII and NlaIII, dNTPs, and DNA ladder were purchased from Fermentas Life Sciences, Vilnius, Lithuania. Forward and reverse primers were obtained from Metabion International AG, Martinsried, Germany through local authorized agency. All other chemicals required for the study were procured from Sigma-Aldrich, St. Louis, MO, USA.

Selection of Subjects, Blood Collection, and DNA Isolation

The study was performed in 332 age-matched case controls (males/females) and 89 PD patients (males/females). The mean age of patients and case controls was 66 and 62 years, respectively. Blood was collected by the clinical experts, authorized for the same. Patients were diagnosed on the basis of the diagnosis criteria suggested by the UK Parkinson's disease society brain bank. While patients were diagnosed on the basis of the presence of cardinal features of PD, controls did not have any clinical history or symptoms of any neurological or non-neurological diseases. PD patients were clinically identified by medical practitioners employing the criteria of disease diagnosis and principle of PD rating scale. Blood was drawn through venous puncture and mixed well in vials containing tri-sodium citrate (3.8 %*w*/*v*; pH 6.5) as an anticoagulant (9:1 ratio). Owing to common usage in genetic and non-genetic studies [18–21], easy accessibility, cost effectiveness, and lack of interaction with the biochemical indexes evaluated in the study, tri-sodium citrate was preferred over other anticoagulants. Blood sample was processed immediately or stored at −80 °C till further use as per the experimental requirement. Genomic DNA was extracted from whole blood using salting out procedure, as described previously [18, 19]. Genomic DNA was precipitated from aqueous phase using absolute ethanol and was quantified by taking the optical density at 260 nm. Calculating the ratio of absorbance at 260 to 280 nm checked the purity of DNA. Agarose gel electrophoresis (0.8 %) was used to check the integrity of genomic DNA. Genomic DNA was used to amplify the desired gene segment using polymerase chain reaction (PCR).

PCR and SNP Genotyping

Genotyping of nNOS exons 18 and 29 was performed employing PCR restriction fragment length polymorphism followed by non-denaturing polyacrylamide gel electrophoresis. Forward and reverse primers and amplification (Mastercycler Gradient Thermo cycler, Eppendorf, Germany) conditions were used, as reported elsewhere [16, 17]. Size of PCR product in the former case was 179 bp while in the later case it was 187 bp. PCR product of nNOS exon 18 was digested with fast digest DraIII restriction enzyme. The digested PCR product was separated in 12.5 % polyacrylamide gel. Gel was stained with ethidium bromide, and image was captured by gel documentation system coupled with UV transilluminator (Alpha Imager System, Alpha Innotech Corporation, San Leandro, CA, USA).

Polymorphonuclear Leukocytes (PMNs) Isolation

PMNs were isolated from freshly collected whole blood using dextran sedimentation, followed by histopaque density gradient centrifugation, as described elsewhere [18, 22, 23]. In brief, PMNs were isolated from buffy coat, which was obtained from the interface of platelet containing plasma and red blood cells after centrifugation of whole blood mixed with anticoagulant. Buffy coat was then mixed with 6 % dextran and allowed to settle the contaminating red blood cells. Upper layer was taken and centrifuged, and pellet containing PMNs was subjected to hypotonic lysis with water to remove contaminating red blood cells. The PMNs present in the pellet were purified with histopaque density gradient centrifugation. PMNs were recovered from the boundary of histopaque 1077-1/1119-1 and were washed thrice with Hank's balanced salt solution buffer [25 mM

containing calcium chloride (1.0 mM) and glucose (10 mM)]. Viability of PMNs was tested by trypan blue exclusion test, which was never less than 95 %.

Estimations of Protein, Nitrite, and Lipid Peroxidation

PMNs were lysed and supernatant was collected to measure the protein, nitrite, and lipid peroxidation. Protein was measured using bovine serum albumin as a standard [24]. Nitrite was estimated in the supernatant using standard procedure [8, 25]. Nitrite content was calculated employing a standard curve of sodium nitrite. On the other hand, lipid peroxidation was measured using Ohkawa et al. method [26] with slight modifications, as described elsewhere [8]. Lipid peroxidation was calculated in terms of nmole malonaldehyde/mg protein.

Statistical Analysis

Allelic and genotypic frequencies were calculated as reported previously [19]. In brief, unadjusted odds ratio (OR) was calculated by 2×2 contingency table with 95 % confidence intervals. Statistical significance was calculated with chi-square test using Epi Info-5 software. Minimum detectable OR with 80 % power significance level (0.05) and existing percent of wild type controls were considered 0.5 and 2.0, respectively, for risk and protection.

Results

Allelic and Genotypic Frequency

The nNOS exon 29 polymorphism [OR for carriers of the T allele=1.58; 95 %; CI=(1.10–2.28), *P* value; 0.0141] showed significant association with PD risk. OR for C/T genotype carriers as compared with reference (C/C) was 1.55 [95 % CI=(0.94–2.55), *P* value; 0.0902]. Similarly, OR for T/T was 2.20 [95 % CI=(1.08–5.34), *P* value; 0.040] and C/T and T/T together was 1.680 [95 % CI=(1.05–2.69), *P* value; 0.031] (Table 1). Genotypic distribution of nNOS exon 29 was different in patients from controls (*P* value; 0.0141). Combined homozygous and heterozygous variants (T/T and C/T) were associated with PD risk. However, genotypic and allelic frequencies of exon 18 nNOS polymorphism did not exhibit significant association with PD risk (Table 2).

Nitrite Content and Lipid Peroxidation

An increase in nitrite content was observed in PD patients as compared with controls irrespective of nNOS exon 29 genotype. Heterozygous variant of nNOS exon 18 also exhibited an increase in nitrite content as compared with control (Fig. 1a, b).

Lipid peroxidation was significantly increased in the PMNs of PD patients of both polymorphic groups irrespective of their genotype as compared with respective controls (Fig. 2a, b).

Discussion

Polymorphism of nNOS gene alters the function of its protein product and finally the extent of NO production. The nNOS 29 polymorphic site, located in an exon of 5′ untranslated region, is often associated with regulation of transcripts and mRNA stability while nNOS 18 polymorphic site, located in the regulatory region, mediates the end effect [16, 27]. Interaction of nNOS and iNOS with environmental risk factors associated with PD is well known [11]. A statistically significant association of nNOS exon 29 polymorphism and lack of any association of nNOS exon 18 polymorphism with PD risk were observed in the study. Results demonstrate that NOS1 could play a decisive role in PD risk among the north Indians. The

Table 1 Summary of the number of cases and controls and nNOS exon 29 allelic and genotypic frequency along with OR and *P* value

	Patients	Case controls	OR (95 % CI, value up to only two digits after decimal)	*P* value
Total patients/case controls	*n*=89	332		
Allele frequency (total number of alleles)				
C	0.66 (118)	0.76 (502)	1.00 (Reference)	
T	0.34 (60)	0.24 (162)	1.58 (1.10–2.28)	0.014
Genotypic frequency (total number of genotypes)				
C/C	0.45 (40)	0.88 (192)	1.00 (Reference)	
C/T	0.43 (38)	0.36 (118)	1.55 (0.94–2.55)	0.090
T/T	0.12 (11)	0.07 (22)	2.20 (1.08–5.34)	0.040
C/T+T/T	0.55 (49)	0.42 (140)	1.68 (1.05–2.69)	0.031

Table 2 Summary of the number of cases and controls and nNOS exon 18 allelic and genotypic frequency along with OR and *P* value

Total patients/case controls	Patients $n=89$	Case controls $n=332$	OR (95 % CI, value up to only two digits after decimal)	*P* value
Allele frequency (total number of alleles)				
C	0.67 (119)	0.73 (486)	1.00 (Reference)	
T	0.33 (59)	0.27 (178)	1.35 (0.94–1.93)	0.098
Genotypic frequency (total number of genotypes)				
C/C	0.47 (42)	0.54 (180)	1.00 (Reference)	
C/T	0.39 (35)	0.38 (126)	1.19 (0.72–1.97)	0.499
T/T	0.14 (12)	0.08 (26)	1.97 (0.89–4.20)	0.090
C/T+T/T	0.53 (47)	0.46 (155)	1.30 (0.81–2.08)	0.275

trend was in accordance with a previous study conducted in another population [16]. Since NO gets converted into nitrite [3, 7, 9], level of nitrite was also measured in cases and controls in order to correlate it with NOS1 polymorphism. Nitrite content was increased in PD patients that carry any genotype of nNOS exon 29 or heterozygous variant genotype of exon 18 as compared with respective controls. This is in accordance with the previous studies that have shown an increased nitrite content and nNOS activity in the PMNs of PD patients [28, 29]. Such observations suggest that nNOS exon 29 polymorphism and heterozygous variant of exon 18 could be associated with

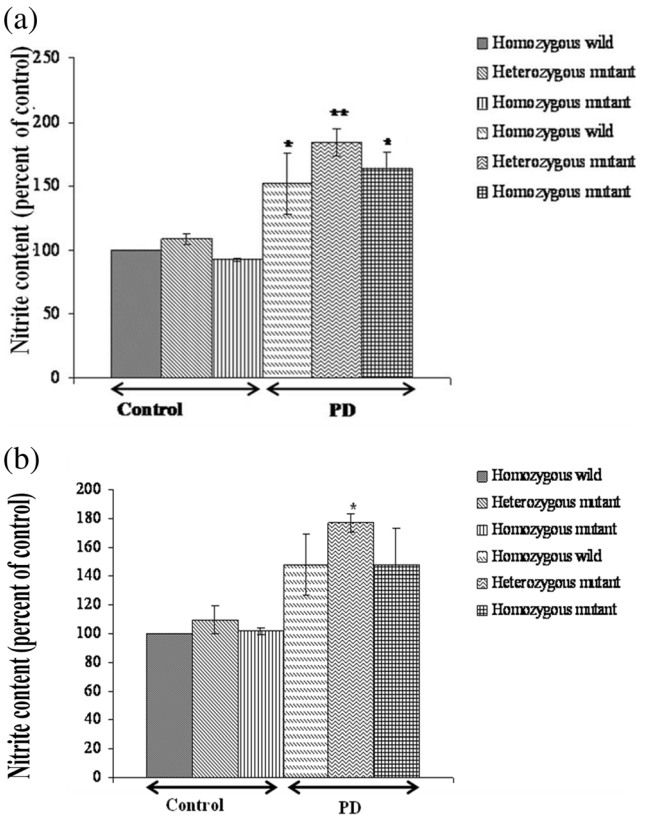

Fig. 1 Effect of nNOS exon 29 (**a**) and exon 18 (**b**) polymorphisms on nitrite content in the PMNs of case controls and PD patients of various genotype carriers. All control values were considered 100 %, and other values were calculated accordingly. Data were analyzed employing one-way analysis of variance along with Newman-Keuls post hoc test. The data are expressed in mean±SEM. Significant changes **P*<0.05 and ***P*<0.01 are expressed as compared with CC wild type of case controls

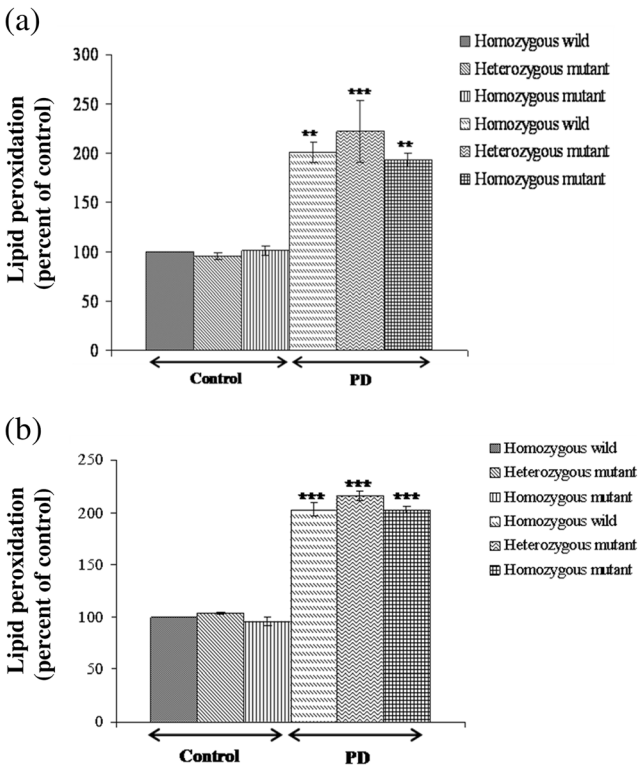

Fig. 2 Effect of nNOS exon 29 (**a**) and exon 18 (**b**) polymorphisms on lipid peroxidation in PMNs of case controls and PD patients of various genotype carriers. All control values were considered 100 %, and other values were calculated accordingly. Data were analyzed employing one way analysis of variance along with Newman-Keuls post hoc test. The data are expressed in mean±SEM. Significant changes ***P*<0.01 and ****P*<0.001 are expressed as compared with CC wild type of case controls

PD risk. An increase in lipid peroxidation in all PD patients was observed as compared with controls. This is in accordance with a previous study, which has shown an increase in lipid peroxidation in PD patients [30].

NO induces oxidative degradation of lipid owing to an increase in level of NOS thereby increasing NO production in Parkinsonism [8]. Statistically significant association of nNOS exon 29 heterozygous variant or combined variants with PD risk could be due to small sample size, and overall biological significance could be negligible as reflected in nitrite content and lipid peroxidation. Altered NOS activity in PD patients as compared with controls suggests that nNOS activity could be regulated by some other polymorphic site(s), which need to be deciphered and not because of the studied sites. Overall, the study demonstrated that probably, polymorphism in nNOS exon 29/18 is not associated with PD risk in north Indian population.

Acknowledgments We thank the Council of Scientific and Industrial Research (CSIR), New Delhi, India for providing the financial support for the study (through Network project BSC0115/miND) as well as senior research fellowship to Satya Prakash Gupta. The CSIR-IITR communication number of this article is 3282.

Conflict of Interest The authors declare that they have no competing interest.

Compliance with Ethical Standards The work was initiated after the approval of the medical ethics committee. Written pre-informed consent was obtained from healthy controls and PD patients prior to blood collection.

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