Türk Biyokimya Dergisi [Turkish Journal of Biochemistry - Turk J Biochem] 2003; 28 (4); 246-251.

Araştırma Makalesi [Research Article]

Yayın tarihi Aralık, 2003 © TurkJBiochem.com

[Published online December, 2003]

Mitochondrial Complex I and IV Dysfunction of Leukocytes in Parkinson's Disease

[Parkinson Hastalığında Lökositlerde Mitokondriyel Kompleks I ve IV İşlevsizliği]

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Kayıt tarihi 25.08.2003; kabul tarihi 20.12.2003
[Received 25.08.2003; accepted 20.12.2003]

ABSTRACT

Mitochondrial dysfunction that generates oxidative stress contributes to the etiology of idiopathic Parkinson's disease (PD). There are contraversial studies on the decrease in complex I activity in peripheral tissues of idiopathic PD, since methodological factors influence these enzyme activities. In order to eliminate these factors, in this study, we purified mitochondria from leukocytes of idiopathic PD patients and analyzed mitochondrial complex I and complex I V enzyme activities in these purified mitochondrial suspensions to evaluate the functional activity of the mitochondrial respiratory enzymes. In addition, ND2 subunit of complex I enzyme were analyzed to identify 5460G/A polymorphism in both leukocytes and platelets of thirthyseven Turkish idiopathic PD patients and 100 healthy subjects. We found a statistically significant decrease in complex I (55 %) and complex IV (58 %) enzyme activities in the leukocytes of idiopathic PD patients. But, there was no significant correlation between these enzyme activities and age, age of onset and duration of the disease. The frequency of 5460G/A polymorphism was found to be 0.08 (3/37) in the idiopathic PD patients and 0.10 (10/100) in the control group. Thus, no effect of ND2 G5460A genotype on complex I enzyme activity was detected. The observed respiratory chain enzyme deficiency supports the hypothesis that systemic mitochondrial dysfunction is important in the pathogenesis of idiopathic PD.

Key Words: Parkinson's disease, complex I, complex IV, mitochondrial DNA

ÖZET

Mitokondrinin işlevsel bozukluğuna bağlı oluşan oksidatif stres idiyopatik Parkinson hastalığının (PD) etiyolojisine katkıda bulunur. Kullanılan yöntem enzim aktivitesini etkilediğinden, idiyopatik PD hastalığında dokuların kompleks I aktivitesindeki azalma ile ilgili çelişkili yayınlar bulunmaktadır. Bu çalışmada, yöntemlerin etkilerini yoketmek için, PD'li hastaların lökositlerinden mitokondriler saflaştırıldı ve mitokondriyel solunum zincirinin işlevsel durumunu belirlemek için kompleks I ve IV'ün aktiviteleri bu süspansiyonlarda ölçüldü. Ayrıca, kompleks I'in ND2 altbirimi, 5460G/A polimorfizmi açısından, 37 idiyopatik PD'li Türk hasta ve 100 sağlıklı bireyin hem lökosit hem de platelet mitokondiyel DNA'larında tarandı. Idiyopatik PD hastalarının lökosit mitokondrilerinde, kompleks I (%55) ve kompleks IV (%58) enzim aktivitelerinde istatiksel olarak anlamlı bir azalma gözlendi ancak, bu azalma ile yaş, hastalığın ortaya çıkma zamanı ve süresi ile arasında bir korelasyon bulunamadı. 5460G/A polimorfizminin sıklığı, idiyopatik PD'li hasta grubunda 0.08 (3/37); sağlıklı grupta ise 0.10 (10/100) bulundu. Bu sonuç, ND2 G5460A genotipinin kompleks I aktivitesi üzerine hiçbir etkisinin olmadığını göstermektedir. İdiyopatik PD'nin patogenezinde, sistemik mitokondriyel işlevsizliğinin önemli olduğu hipotezini, solunum zinciri enzim eksikliği bulgusu, desteklemektedir.

Anahtar Kelimeler: Parkinson hastalığı, kompleks I, kompleks IV, mitokondriyel DNA

INTRODUCTION

Parkinson's disease (PD) is a chronic progressive neurodegenerative movement disorder. Tremors, rigidity, postural instability and bradykinesia are characteristic symptoms of PD. Idiopathic PD is the most common form of parkinsonism, but its etiology is still unknown. Several factors, however like oxidative stress, accelerated aging, environmental toxins, and genetic predisposition has been implicated in the etiology of idiopathic PD. It has been shown that rotenone and N-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) toxins that inhibit complex I activity in the mitochondrial electron transport system induce neurologic disorders like PD. Since then, mitochondrial basis of PD has began to be searched [1-4].

The oxidative phosphorylation system consists of five enzyme complexes (complexes I-V), which are located in the inner mitochondrial membrane. Among these, complex I is the largest enzyme that composed of about 43 subunits, 7 of which (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) are encoded by the mitochondrial DNA (mtDNA). It has been shown that a defect in complex I activity is systemic in idiopathic PD since it affects tissues outside the brain such as platelets and muscle [1,5-8]. However, to date, decreased mitochondrial complex I and complex IV activities in purified mitochondria from lymphocyte of idiopathic PD patients has not been found. Yoshino et al. reported no decrease in complex I and in complex IV activities while Barroso et al. found decrease in activities of complex I and complex IV in lymphocyte homogenates of PD patients. Martin et al. repeated the same experiments while isolating mitochondria from lymphocytes of PD patients and found that none of the enzyme activities were significantly different from those in controls [9-11]. Knowing that methodological factors affect those enzyme activities and in order to eliminate these factors, in this study, we purified mitochondria from leukocytes of idiopathic PD patients and analyzed mitochondrial complex I and complex IV enzyme activities in these purified mitochondrial suspensions.

Cytoplasmic cybrid studies have shown that idiopathic PD patients may express mutations in subunits of complex I-encoding mtDNA. Since cells with mtDNA from idiopathic PD patients maintained the decreased complex I activity, the reduced complex I activity may be due to mutations in the mitochondrial genome [12,13]. Therefore, in this study, idiopathic PD patients (n=37) and 100 healthy subjects were screened for G5460A polymorphism in the ND2 subunit of complex I.

MATERIALS AND METHODS

Patients and controls

Thirthy-seven patients with idiopathic PD at the Department of Neurology at Hacettepe University, Faculty of Medicine, and 17 age-matched control group without known neurological disorders were evaluated for measurement of mitochondrial enzyme activities. As a control group for screening of G5460A polymorphism we evaluated 100 healthy volunteers. All patients were under antiparkinsonian-medication of levo-dopa, selegiline and pergolide. The clinical backgrounds of the subjects are shown in Table 1.

Table 1 Clinical information about idiopathic PD patients and control subjects

	Idiopathic PD patients	Control (for enzyme activities)	Control (for G5460A polymorphism)
No of patients	37	17	100
Age (years)	57.1 ± 14.5	59.3 ± 12.0	50.0 ± 11.1
Age of onset(years)	50.4 ± 15.2		
Duration of the disease (years)	6.7 ± 3.9		
H&Y Scale	2.3 ± 0.7		

Isolation of leukocytes and platelets

Venous blood samples (20 ml each) from healthy subjects and patients with idiopathic Parkinson disease were collected in EDTA-containing tubes and centrifuged at 1800 x g for 15 min. After the centrifugation, the supernatant and mid layer were used for platelet and leukocyte isolation, respectively. The supernatant was collected and centrifuged with Sorvall SS-34 rotor at 14500 x g for 20 min. Platelet rich pellets were suspended in 150 mM KCl, 50 mM Tris-HCl, 1 mM EDTA, pH 7.4 buffer and used for mtDNA isolation. Leukocytes were isolated using Histopaque-1119 (Sigma) according to the manufacturer's procedure.

Complex I and Complex IV enzyme assays

The leukocyte mitochondria were isolated following the method of Pich et al. [14] with the following modifications: In order to obtain mitochondria, the leukocyte suspensions from control and PD patients were homogenized with a glass-teflon homogenizer. The homogenate was centrifuged at 6000 x g for 10 min at 4°C. The mitochondria were collected from the supernatant by centrifugation at 20800 x g for 20 min, and the pellet was resuspended in 150 mM KCl, 50 mM Tris-HCl, 1 mM EDTA, pH 7.4. The mitochondrial suspension was sonicated at 60 watts for five times (10 s-periods with 50 s-intervals). The mitochondrial membrane fragments were diluted 1:1 in 0.25 M sucrose, 1.0 mM EDTA, 30 mM Tris/HCL, pH 7.7, and centrifuged at 33000 x g for 10 min. The supernatant was then ultracentrifuged at 100000 x g for 60 min and the pellet was resuspended in the same buffer. The final suspension was assayed for complex I and complex IV enzyme activities.

Complex I activity was measured at 340 nm by monitoring the oxidation of NADH for 3 min. The rotenone insensitive form of the complex I was calculated [15].

Complex IV activity was measured by monitoring the oxidation of reduced cytochrome c at 550 nm [15]. Protein concentrations were determined by Lowry method [16].

All spectrophotometric assays were performed on a Milton Roy Spectronic 3000 Array spectrophotometer.

Lactate Dehydrogenase Enzyme Assay

Lactate dehydrogenase activity was measured at 340 nm according to the method of Beutler [17].

Statistical analysis

The results were analyzed statistically by one-way analysis of variance (ANOVA).

DNA extraction and Polymerase chain reaction

Genomic DNA was extracted from leukocytes using QiampDNA extraction mini kit (Qiagen). Platelet mtDNA was purified with the same procedure. The 302 bp of mtDNA-ND2 region was amplified using primers and annealing conditions as previously described [18]. DNA products were verified by electrophoresis on a 2 % agarose-1XTAE gel and analysed after staining with ethidium bromide.

Restriction enzyme digestion

MtDNA-ND2 PCR products were digested with HphI restriction enzyme at 37°C for 16 hours incubation [18].

RESULTS

During purified mitochondrial preparations from leukocytes of idiopathic PD patients to measure complex I and complex IV enzyme activities, we measured lactate dehydrogenase enzyme activity as a cytosolic marker. Each enzyme activity assay was carried out at least three times on each sample to establish the linearity of the assay. Intravariability between triplicate values was between 15-20 %.

The results of complex I and complex IV activities for idiopathic PD patients and control subjects are given in Table 2. We have analyzed 37 idiopathic PD patients and found that 81 % of these patients were clearly out of the activity range of the control group and had low complex I activity (Fig. 1A). For the accurate evaluation of complex I activity belong to inner mitochondrial membrane, the samples with rotenone sensitivity ≥74 % were considered. The mean activity of complex I was 55 % of that of the control (Table 2). In addition, there was a significant decrease (58 %) in the leukocyte complex IV activitiy in idiopathic PD patients (Table 2). Although it seemed to be an overlap between idiopathic PD patients and control group (Fig. 1B), 34% of idiopathic PD patients were out of complex IV activity range and the rest of these patients' complex IV activities remained in the lower half of the control values. The relationship between complex I and IV enzyme activities and age of the patients, age of the onset and the duration of the disease were analyzed. No significant correlation between age, age of onset, disease duration and enzyme activities were found in idiopathic PD patients (Fig. 2 and Fig. 3).

Table 2 Complex I and Complex IV activities in idiopathic PD patients and control subjects

	Control (n= 17)	Idiopathic PD (n=37)
Complex I Activity (U/mg protein)	15.80 ± 2.76	*7.08 ± 3.74
Complex IV Activity (U/mg protein)	13.25 ± 8.95	*5.47 ± 3.07

The mean ± SD values were calculated from three independent experiments.
*p<0.001

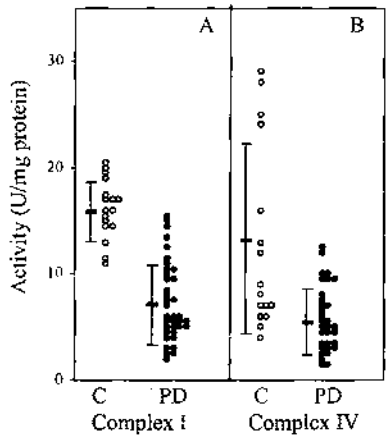

Figure 1 Complex I and Complex IV activities in idiopathic PD patients and controls. Dots represent individual subjects, bars are standard deviations and lines are mean values.

In this study, idiopathic PD patients were evaluated for G5460A polymorphism. Restriction pattern of 302 bp of ND2 PCR product by *HphI* is shown in Figure 4. The frequency of heteroplasmic G5460A polymorphism in the ND2 subunit of complex I was found to be 0.08 (3/37) in idiopathic PD patients and 0.10 (10/100) in control group ($p > 0.05$). We found the same frequency of G5460A polymorphism in both platelet and leukocyte of patients and control.

DISCUSSION

The mitochondrial respiratory chain function has become an important issue in PD because of MPTP and rotenone-induced parkinsonism. Since the reduced complex I activity, that is one of the basic abnormalities responsible for mitochondrial dysfunction, has been reported in PD not only in the substantia nigra [19,20] but also in the skeletal muscle [6-8] and the platelets [5,21,22], it has been accepted that systemic mitochondrial dysfunc-

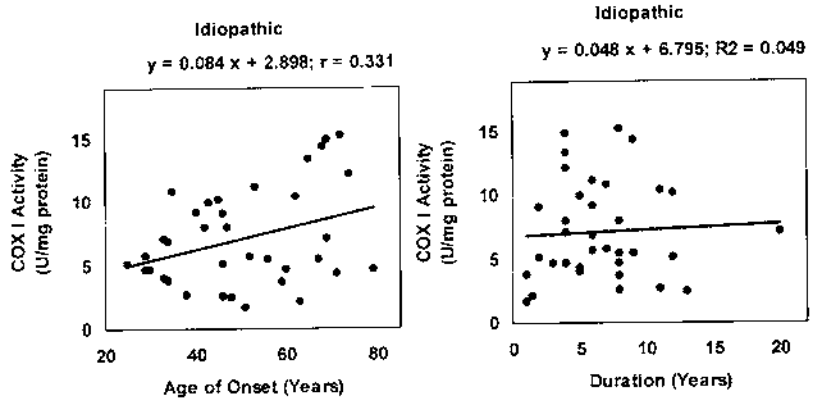

Figure 2 The relationship between the age of onset, duration of the disease and the activities of Complex I in leukocytes.

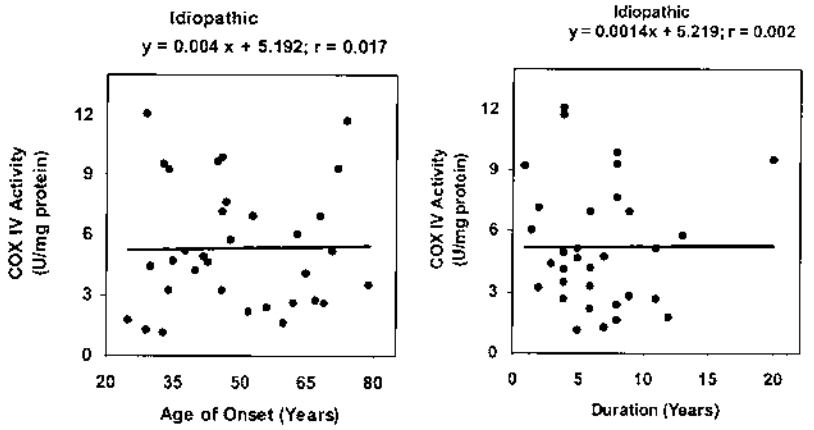

Figure 3 The relationship between the age of onset, duration of the disease and the activities of Complex IV in leukocytes.

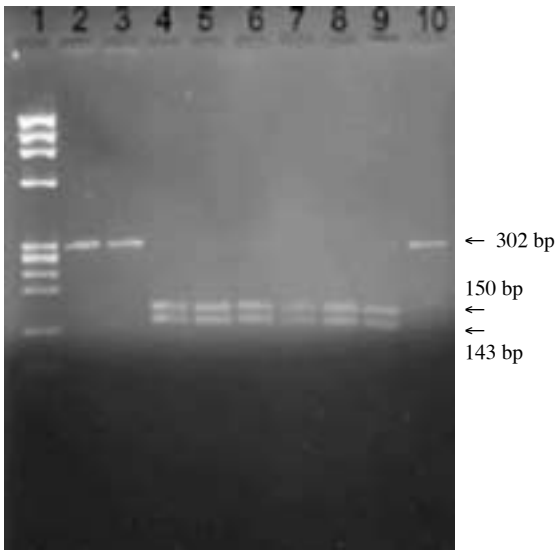

Figure 4 Restriction pattern of 302 bp of ND2 PCR product by *HphI* . Lane 1 contains the marker fX174. Lane 2, 3, 10 show undigested PCR product. After the cleavage with restriction enzyme, the PCR product in lane 4-9 is digested yielding two bands (159 bp and 143 bp), indicating G5460A polymorphism.

tion is important in the pathogenesis of idiopathic PD. Although many studies have supported the decrease in complex I activity in peripheral tissues of idiopathic PD, there are also contraversing studies [9,10,23].

In literature, there are two studies that have pointed out normal complex I and complex IV activities in lymphocytes of idiopathic PD patients [9,10] while there is only one study reporting reduced activity [11]. On the other hand, almost all studies in platelets showed between 25-50 % decrease in complex I enzyme activity [5,21,22]. The decrease in mitochondrial enzyme activities in platelets should be almost the same as in the leukocytes. This difference may be caused by the methodological factors. Since the amount of the mitochondrial enzymes in the platelets and leukocytes is relatively low, and this enzyme activities are affected by various factors, care must be taken while isolating mitochondria and measuring enzyme activities. In this study, we purified mitochondria from leukocytes and analyzed complex I and complex IV enzyme activities in the leukocyte of idiopathic PD patients. We found a statistically significant decrease in complex I (55 %) and IV (58 %) enzyme activities but this defect in activities did not correlate with the age, age of onset and the duration of the disease. However, Martin et al. reported normal complex I and IV activities in isolated mitochondria from lymphocytes of untreated PD patients [10]. In this study, all idiopathic PD patients were receiving L-dopa at the time of enzyme activity measurements. It has been shown that L-dopa treatment does not affect mitochondrial electron transport chain enzymes [7,8,21,24]. Thus, the decrease

in complex I and IV activities are related to idiopathic PD rather than to the treatment with L-dopa. This study shows that not only complex I defect in idiopathic PD is systemic but also complex IV defect appears to be systemic.

What could be possible causes for the decrease in complex I activity in idiopathic PD patients? To address this question, many researchers are seeking for possible answers by digging genetic and biological pathways. Cytoplasmic cybrid studies have demonstrated that complex I deficiency resulted in mutations in the mitochondrial genome [12,13]. MtDNA has been searched by several groups to identify a specific mutation that correlate with complex I deficiency in PD [25-29]. To date, however no specific mutation for idiopathic PD has been detected. An increased frequency of heteroplasmic G5460A transition that substitutes threonine for alanine has been reported in substantia nigra of PD patients [30,31]. Therefore, we have screened mtDNA for heteroplasmic G5460A polymorphism in the ND2 subunit of complex I in both platelets and leukocytes of idiopathic PD patients and that of control group. Our results showed that ND2 G5460A genotype was almost in same frequency in idiopathic PD patients and control group. Thus, no effect of ND2 G5460A genotype on complex I enzyme activity was detected. Indeed, this result might be expected. The mutated mtDNA may not be accumulated, since both the lymphocytes and platelets have a rapid turnover. Since tRNAs are normally synthesized in excess of minimum required for normal mitochondrial protein synthesis, the remaining normal tRNAs complements the function of mutated ones. Therefore, very high proportion of mtDNA is required for many tRNA point mutations in order to determine oxidative phosphorylation enzyme defects. On the other hand, mutations in mtDNA genes encoding complex I, III, IV and V subunits seem to cause enzymatic defects at low levels of heteroplasmy [32].

Our results correlate well with the results of Bandmann et al. (1997), Simon et al. (2000) and Richter et al. (2002) that do not support an increased frequency of G5460A polymorphism in PD patients. We found that the frequency of heteroplasmic G5460A polymorphism was 0.08 in both the leukocytes and the platelets of idiopathic PD patients and 0.10 in control group. In addition, Bandmann et al. reported that the G5460A polymorphism was present with identical frequency in patients brain (0.06) and control (0.06) [29]. Richter et al. has also reported that in PD patients platelets, the G5460A polymorphism was present in the same frequency as in control [28].

Acknowledgments

This work was supported by the Scientific and Technical Research Council of Turkey (Grant No. SBAG-2596 and Grant No. TBAG-1754) and by Hacettepe University Scientific Research Unit (Grant No. 0002101001).

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