DOI 10.1007/s00702-004-0195-y
J Neural Transm (2005) 112: 499–518

Journal of Neural Transmission
Printed in Austria

Re-evaluation of the dysfunction of mitochondrial respiratory chain in skeletal muscle of patients with Parkinson's disease

K. Winkler-Stuck[1], **E. Kirches**[2], **C. Mawrin**[2], **K. Dietzmann**[2], **H. Lins**[1], **C.-W. Wallesch**[1], **W. S. Kunz**[3], and **F. R. Wiedemann**[1]

[1] Klinik für Neurologie, and
[2] Institut für Neuropathologie, Otto-von-Guericke-Universität, Magdeburg, and
[3] Klinik für Epileptologie, Universität Bonn, Germany

Received February 25, 2004; accepted June 26, 2004
Published online August 3, 2004;

Summary. The origin and tissue distribution of the mitochondrial dysfunction in Parkinson's disease (PD) remains still a matter of controversy. To re-evaluate a probably free radical-born, generalized mitochondrial impairment in PD, we applied optimized enzymatic assays, high resolution oxygraphic measurements of permeabilized muscle fibers, and application of metabolic control analysis to skeletal muscle samples of 19 PD patients and 36 age-matched controls. We detected decreased activities of respiratory chain complexes I and IV being accompanied by increased flux control coefficients of complexes I and IV on oxygen consumption of muscle fibers. We further investigated if randomly distributed point mutations in two discrete regions of the mitochondrial DNA are increased in PD muscle, and if they could contribute to the mitochondrial impairment. Our data confirm the previously debated presence of a mild mitochondrial defect in skeletal muscle of patients with PD which is accompanied with an about 1.5 to 2-fold increase of point mutated mtDNA.

Keywords: Mitochondria, mitochondrial DNA, oxidative phosphorylation, Parkinson's disease, skeletal muscle.

Introduction

Parkinson's disease (PD) is a neurodegenerative disease affecting the dopaminergic neurons of the substantia nigra. The etiology of the disease is unknown in the majority of cases, except some rare familial forms of PD [for review, see Gasser (2001)]. More than a decade ago, it has been discovered that mitochondria play an important role in the process of neurodegeneration, mainly on the basis of findings of a mitochondrial respiratory chain complex I (NADH:

ubiquinone oxidoreductase) deficiency in postmortem brain tissue of PD patients (Mizuno et al., 1989; Schapira et al., 1989), together with reduced immunoreactivity for complex I (Hattori et al., 1991). The origin of this well-established complex I deficiency could not be determined so far, neither on molecular genetic nor on protein structural level.

It has been shown over the past years that free radicals play a crucial role in the process of neurodegeneration [for review, see Jenner (2003)]. If a disturbtion of the fine-tuned balance of free radical production and detoxification is pathogenic in PD, then it has to be clarified whether a mitochondrial dysfunction is restricted to specific structures in the brain, or (as a result of a *generalized* imbalance of free radicals) if mitochondria in other cells also show any involvement. These considerations are not novel: A decade ago it was already assumed that a mitochondrial dysfunction in PD might be present also in other tissues than brain (Bindoff et al., 1989; Shoffner et al., 1991; Nakagawa-Hattori et al., 1992). However, we found enormous inconsistencies regarding the expression of defects of respiratory chain enzymes in PD, for example in skeletal muscle (see Table 1). It has to be clarified at least, whether the mitochondrial dysfunction in PD is generalized, or restricted to certain regions of the brain. Using an extensive range of optimized, well-established analytical methods, we tried to reduce uncertainty of previous data obtained on skeletal muscle tissue of PD patients.

The genetic background of a putative deficiency of respiratory chain enzymes remains elusive (Richter et al., 2002). We therefore hypothesized that the ROS-induced damage of the mitochondrial DNA (mtDNA) would possibly result in randomly distributed somatic point mutations, presumably at extremely low levels of heteroplasmy, in all regions of the mitochondrial genome. Therefore we used the ''double PCR and digestion'' method (Pallotti et al., 1996) in two discrete regions of the mitochondrial genome. This is a method which has been proven to detect extremely low levels of point mutated mtDNA (Wiedemann et al., 2002).

Table 1. Results of previous investigations on respiratory chain enzymes in skeletal muscle of PD patients

Authors	Complex I or Complex I + III	Complex II or Complex II + III	Complex IV
Bindoff et al. (1989, 1991)	+	+	+
Shoffner et al. (1991)	+	+	+
Nakagawa-Hattori et al. (1992)	+	−	−
Mann et al. (1992)	−	−	−
Anderson et al. (1993)	−	−	−
Cardellach et al. (1993)	+	−	+
Di Monte et al. (1993)	−	−	−
DiDonato et al. (1993)	−	−	−
Blin et al. (1994)	+	−	+
Reichmann et al. (1994)	−	−	−
Taylor et al. (1994)	−	−	−
Wiedemann et al. (1999)	+	−	+

(+) statistical significant difference to controls, (−) not significant

Preliminary results of the study were already presented in abstract form (Wiedemann et al., 1999).

Material and methods

Patients

PD subjects were selected from volunteers following an appeal of the ''Deutsche Parkinson-Vereinigung''. We selected younger patients without other diseases than PD and without any

Table 2. Parkinson's disease patients characteristics

Patient no.	Sex	Age (years)	Duration of disease (years)	UPDRS motor score	Hoehn/ Yahr score	L-dopa treatment (mg/d)	Muscle histopathology and electron microscopy
1	m	65	8	34	3	1050	Mild to moderate neurogenic changes, dark staining for mitochondrial enzymes. ElMi normal.
2	f	47	10	25	3	250	Mild neurogenic changes, mild myopathic changes. ElMi normal.
3	f	62	5	39	3	0	Moderate neurogenic changes. ElMi normal.
4	f	51	7	15	1.5	50	Mild neurogenic changes, dark staining for mitochondrial enzymes. ElMi normal.
5	f	62	11	13	1.5	300	Mild fiber atrophy. ElMi not performed.
6	m	49	8	35	3	512.5	not determined.
7	m	71	3	8	1	375	Moderate fiber atrophy, enhanced staining for mitochondrial enzymes. ElMi normal.
8	f	64	4	15	2.5	150	Mild neurogenic changes, dark staining for mitochondrial enzymes. ElMi normal.
9	f	47	3	7	1	0	Mild neurogenic changes, dark staining for mitochondrial enzymes. ElMi normal.
10	m	62	10	21	2.5	300	Mild fiber atrophy, myopathic features, rare fiber necrosis, dark staining for mitochondrial enzymes. No inflammation. ElMi not performed.
11	m	78	13	19	2.5	500	Moderate neurogenic changes. ElMi normal.
12	f	37	3	11	2	0	Dark staining for mitochondrial enzymes. ElMi normal.
13	m	56	3	7	1	200	not determined.
14	m	62	2	1	1	300	Moderate neurogenic changes, mild myopathic changes, dark staining for mitochondrial enzymes, ragged red fibers (<1%). ElMi with mitochondrial crystalloid inclusions.
15	m	65	3	16	2.5	0	Mild fiber atrophy, mild myopathic changes, dark staining for mitochondrial enzymes. ElMi not performed.
16	m	71	4	20	3	200	Mild to moderate neurogenic changes, mild myopathic changes. ElMi not performed.
17	m	54	12	29	3	1100	not determined.
18	f	61	5	13	1.5	0	Mild fiber atrophy. ElMi normal.
19	f	42	4	20	2.5	300	Mild to moderate neurogenic changes, mild myopathic changes. ElMi not performed.

m male; *f* female; *ElMi* electron microscopy

history of toxins and neuroleptic medication. 19 patients with PD (9 female and 10 male, age 37–78 years, mean 58 years) underwent skeletal muscle biopsy. The patients suffered from a 2–13 years (median: 4.5) history of PD. The median motor score of the Unified Parkinson's Disease Rating Scale (UPDRS), measured whilst 'on', was 26.5 points (range: 4–57). All patients suffered from the akinetic-rigid type of the disease. There was no family history in any of the PD patients. PD was diagnosed on the basis of clinical evaluation, absence of signs accompanying secondary parkinsonism, and response to dopaminergic drugs. Clinical evaluation of all patients was performed by one senior neurologist (H.L.). Except for one PD patient (No. 7), who was a long-distance cyclist, no patient reported to perform physical endurance training. Patient's characteristics are demonstrated in Table 2.

As *controls for biochemical studies* we selected 36 patients with muscular pain, fatigue, cramps and vague electromyographic abnormalities who underwent diagnostic muscle biopsy. There was no statistical difference between the age of the PD and control subjects (age 31–77 years, mean 52 years). The control subjects did neither have weakness and atrophy nor any sign for a neuromuscular disorder in the routine histopathological investigation and in the routine laboratory investigation (including creatine kinase and lactate). None of the controls was on medication with levodopa, neuroleptics, or other drugs known to affect mitochondrial function. All control subjects were clinically evaluated by the same senior neurologists (C.W. and H.L.). As controls for molecular genetic studies on skeletal muscle we selected 9 age-matched patients (age 47–62 years, mean 57 years) out of the 36 control patients with biochemical data representative for the whole control group. All patients and volunteers gave written informed consent (according to the Declaration of Helsinki) prior to biopsy. The study was approved by the ethics committee of the Medical Faculty, Magdeburg University.

Histological investigation

Staining for HE, cytochrome c oxidase, succinate dehydrogenase, NADH reductase, myofibrillar ATPase, and modified Gomori-Trichrome staining were performed following standard procedures (Sciacco and Bonilla, 1996; Dubowitz and Brooke, 1973).

Preparation of muscle fibers

About 50 mg of biopsy tissue (M. vastus lateralis exclusively) was used for isolation of saponin-permeabilized fibers as previously described (Saks et al., 1998). The saponin treatment was performed as described (Kunz et al., 1993).

Respiration measurements and inhibitor titrations

The oxygen consumption of saponin-permeabilized muscle fibers was analysed at 25°C using the "Oroboros" high resolution oxygraph (Anton Paar, Graz, Austria) as previously described (Kunz et al., 1993; Saks et al., 1998). We used the specific complex I inhibitor amytal and the specific complex IV inhibitor sodium azide to titrate the ADP-stimulated maximal respiration of muscle fibers for determinations of the flux control coefficients (C_i), as described (Kuznetsov et al., 1997; Wiedemann et al., 1998).

Enzyme activities

All tissue samples used for enzymatic assays were kept frozen in liquid nitrogen until analysis. All enzymatic activities were determined at 30°C in tissue homogenates using a Cary 100^{TM} spectrophotometer (Varian, Mulgrave/Victoria, Australia) with two exceptions as stated below. Skeletal muscle homogenate was made from 50 mg of muscle tissue in ice-cold phosphate buffer, using an ultra-turrax homogenizer T25 (IKA, Staufen, Germany) 3 times for 15 s at 24000 rpm, and was centrifuged at 14000 rpm in a Eppendorf 5417R centrifuge at 4°C for 15 min. The CS activity was measured in the supernatant as described in (Bergmeier, 1974). The activities of the respiratory chain enzymes were measured in the remaining pellet (resuspended in ice-cold phosphate buffer). To find out whether the low substrate affinity of human skeletal muscle cytochrome

c oxidase (COX) to the bovine cytochrome c (Van Kuilenburg et al., 1991) leads to uncertain COX activity determinations, we measured the activity simultaneously in duplicate: First, using the "classical" COX assay with a bovine cytochrome c concentration of 80 μM (Wharton and Tzagoloff, 1967) and second, using an optimized spectrophotometric method (Wiedemann et al., 2000), with a dual wavelength spectrophotometer (Aminco DW 2000^{TM}, SLM Instruments, Rochester/NY, U.S.A.), the wavelength pair 510 nm–535 nm ($\varepsilon_{red\text{-}ox} = 5.9\,mM^{-1}cm^{-1}$) and in the presence of 200 μM bovine cytochrome. The activity of rotenone-sensitive NADH:CoQ-reductase was measured as described in (Wiedemann et al., 2000). The succinate: cytochrome c reductase was measured at 550 nm after preactivation for 5 min with 20 mM succinate in 100 mM phosphate buffer (pH 7.4) containing 1 mM KCN and 80 μM ferrocytochrome c. All enzymatic activities were assessed at least in duplicate.

Double PCR and digestion (DPD)

The method has been described previously (Pallotti et al., 1996; Wiedemann et al., 2002). Total DNA was extracted from frozen skeletal muscle tissue by proteinase-K digestion and phenol-chloroform extraction. Two mtDNA polymerase chain reaction (PCR) fragments were amplified with 6-FAM labeled forward primers: One encompassing the mtDNA region of the nucleotides (nt) 7440–7445 (the recognition sequence for the restriction endonuclease *XbaI*), the other encompassing the mtDNA region nt 8991–8995 [the recognition sequence for *MvaI*; numbering according to (Anderson et al., 1981)]. Both regions can harbour well-established pathogenic mtDNA mutations: nt 7445 is mutated in sensorineural hearing loss [SNHL; Reid et al. (1994)], and nt 8993 is mutated in neuropathy, ataxia, and retinitis pigmentosa [NARP; Holt et al. (1990)]. PCR products were digested with 0.2 U/ng DNA overnight with *MvaI* (restriction site at nt 8993) or *XbaI* (restriction site at nt 7445); both enzymes cut only wild type mtDNA at these respective sites. Complete digestion was assured by separation of the 6-FAM labeled PCR products on an ABI Prism® 310 capillary sequencer as follows below. The digested DNA samples were used as a template for a second round of PCR. Since only uncut molecules could be re-amplified, mutated (i.e. uncuttable) mtDNA molecules were amplified selectively. After a second round of digestion with either *MvaI* or *XbaI*, 10 μl of the digestion product were electrophoresed through a polyacrylamide gel (5.8% for the fragment containing the *XbaI* site and 8% for the fragment containing the *MvaI* site) in a horizontal electrophoresis device (Multiphore 2^{TM}, Pharmacia, Uppsala, Sweden) and silverstained to estimate DNA content and quality. The digested and undigested DNA fragments were separated on an ABI Prism® 310 capillary sequencer (Applied Biosystems, Foster City/CA, U.S.A.), and the undigested DNA was quantified with respect to the total amount of PCR fragment amplified by means of the GeneScan® 3.1.2 Analysis Software (Applied Biosystems). The GeneScan®-500 Rox^{TM} Size Standard (Applied Biosystems, Warrington, U.K.) was used for the identification of the two 6-FAM labeled fragments.

PCR-conditions

After denaturation for 3 minutes at 94°C, 30 cycles were performed including denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C followed by a final extension step at 72°C for 7 minutes. The digestion time was 16 hours for both enzymes. The primers for the fragment containing the *XbaI* site were nt 7321–7340 and nt 7669–7648, according to the Cambridge sequence (Anderson et al., 1981). For the fragment containing the *MvaI* site we used nt 8657–8674 and nt 9279–9257.

Southern blotting

The procedures were described previously (Kirches et al., 1998). The blots were hybridized with a digoxygenin-labeled total human mtDNA probe (DNA extracted from isolated skeletal muscle mitochondria). Digoxygenin detection was performed using an anti-digoxygenin antibody, conjugated with alkaline phosphatase and CSPD-chemiluminescence (Tropix, Bedford/MA, U.S.A.). Screening for mtDNA depletion was performed using a mixed probe, which contained a P^{32}-labeled, cloned fragment of the human 18S-rRNA-gene, besides the P^{32}-labeled mtDNA.

The plasmid was a kind gift from Dr. C. McMillan (Montreal, Kanada). Labeling of equal amounts of *PvuII*-digested human mtDNA and of the plasmid insert was performed using the standard ready-reaction-mix from Amersham (Freiburg, Germany). The hybridized blots were read in a Fujix BAS-1000 phosphoimager (Fuji PhotFilm Co., Tokyo, Japan). The ratio of the total intensities of both fragments (16.6 kb versus 12 kb) was calculated.

Sequencing

Total DNA was extracted from frozen skeletal muscle tissue and from one milliliter of an EDTA blood sample. PCR amplification was performed in a PTC100 thermal cycler (Biozyme, Hessisch-Oldendorf, Germany) starting with 100 ng DNA in a total reaction volume of 25 µl containing 0.3 U Goldstar DNA polymerase (Eurogentec, Herstal, Belgium) in the appropriate buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP and 300 nM of the primers. All 20 base pair (bp) primers were designated according to the position of their 5′-site in the L-strand or H-strand of the Cambridge sequence (Anderson et al., 1981): L4236 + H4480 (tRNA cluster), L4531 + H4920 (ND2). After denaturation for 3 minutes at 93°C, 25 cycles were performed: 93°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, followed by a final extension step at 72°C for 7 minutes. Following electrophoretic control, the fragments were sequenced in both directions on an ABI Prism® 273A sequencer (Applied Biosystems, Foster City/CA, U.S.A.) using the big dye® terminator technology (Applied Biosystems, Warrington, U.K.).

Data analysis

Results are represented as mean values ± standard error of the mean. Statistical significance was assessed by unpaired two-tailed Student's *t*-test for comparison of clinical data, DPD, and enzymatic activities, and by Mann-Whitney-U test for comparison of citrate synthase normalized data. Correlations were verified using Spearman's nonparametric correlation analysis. A p-value of <0.05 was accepted as the level of statistical significance.

Results

Because of the comprehensiveness of the biochemical investigations on limited amounts of biopsy material we could sometimes not apply *all* procedures to *all* specimens. We nevertheless decided to demonstrate here all results to prevent a selection of data and to avoid sharing the subjects into small subgroups. All clinical and biochemical investigations were performed by the same individuals over the whole time period without any changes in the methodology.

Histopathology in skeletal muscle samples

Cryostat sections were processed from 16 biopsy specimens. Table 2 shows that the majority of the patients exhibited slight neurogenic changes (i.e., mild type grouping, moderate fiber atrophy and some ''dark angulated'' fibers). Interestingly, 9 subjects showed very intensive mitochondrial staining in the SDH and NADH dehydrogenase reaction. In one sample (No. 14) we noticed mitochondrial crystalloid inclusions in the electron microscopy and some ragged red fibers (less than 1% in a section). We could not reveal other samples with features of a mitochondrial myopathy (i.e., ''ragged red'' fibers or COX-negative fibers with dark staining for SDH).

Biochemistry in skeletal muscle homogenates

We determined the activities of CS, rotenone-sensitive NADH:cytochrome c reductase (complexes I + III), rotenone-sensitive NADH:CoQ_1 reductase

Table 3. Mitochondrial enzymatic activities in skeletal muscle homogenates of PD patients and controls

PD patient no.	Citrate synthase	NADH: cytochrome c reductase	NADH: CoQ_1 reductase	Succinate: cytochrome c reductase	Cytochrome c oxidase*	Cytochrome c oxidase**
1	22.9 ± 0.08	3.41 ± 0.06	1.70 ± 0.04	1.53 ± 0.07	3.72 ± 0.18	2.80 ± 0.12
2	11.6 ± 0.00	1.95 ± 0.03	1.28 ± 0.04	1.04 ± 0.02	2.10 ± 0.27	4.50 ± 0.35
3	15.8 ± 0.09	1.80 ± 0.04	0.58 ± 0.02	1.96 ± 0.05	1.70 ± 0.01	4.92 ± 0.25
4	17.4 ± 0.05	2.66 ± 0.03	0.90 ± 0.08	2.57 ± 0.02	2.83 ± 0.00	3.10 ± 0.25
5	17.7 ± 0.02	2.35 ± 0.03	0.59 ± 0.07	1.93 ± 0.11	2.29 ± 0.08	3.90 ± 0.14
6	19.5 ± 0.00	3.27 ± 0.06	0.60 ± 0.05	2.84 ± 0.10	5.05 ± 0.01	n.d.
7	25.5 ± 1.39	3.30 ± 0.03	2.51 ± 0.13	3.52 ± 0.39	5.03 ± 0.02	15.90 ± 1.10
8	22.3 ± 0.21	4.83 ± 0.07	n.d.	4.07 ± 0.16	4.99 ± 0.22	n.d.
9	12.8 ± 0.94	2.35 ± 0.01	1.54 ± 0.10	1.53 ± 0.03	1.88 ± 0.09	7.60 ± 0.53
10	22.3 ± 0.10	4.52 ± 0.09	1.70 ± 0.08	2.56 ± 0.24	4.36 ± 0.05	10.80 ± 0.80
11	11.9 ± 0.51	1.69 ± 0.02	0.70 ± 0.08	1.33 ± 0.07	1.20 ± 0.05	n.d.
12	12.1 ± 0.01	2.16 ± 0.02	1.10 ± 0.02	1.56 ± 0.03	3.48 ± 0.15	5.20 ± 0.45
13	18.6 ± 0.45	1.21 ± 0.14	0.78 ± 0.08	2.67 ± 0.05	1.99 ± 0.01	n.d.
14	20.3 ± 0.60	3.65 ± 0.07	2.80 ± 0.11	3.27 ± 0.11	3.21 ± 0.07	8.80 ± 0.66
15	20.7 ± 1.80	1.95 ± 0.10	1.57 ± 0.04	2.13 ± 0.05	6.36 ± 0.55	10.30 ± 0.70
16	9.9 ± 0.12	1.72 ± 0.05	n.d.	1.89 ± 0.06	2.96 ± 0.04	n.d.
17	17.0 ± 0.32	4.79 ± 0.33	2.24 ± 0.03	3.09 ± 0.00	1.37 ± 0.23	11.40 ± 1.20
18	15.6 ± 0.15	1.57 ± 0.05	n.d.	0.99 ± 0.02	2.63 ± 0.29	n.d.
19	12.8 ± 0.10	2.06 ± 0.06	0.76 ± 0.04	1.44 ± 0.02	1.73 ± 0.01	3.60 ± 0.09
Controls (n = 36)	12.6 ± 4.5	2.72 ± 1.31	1.47 ± 0.58	1.79 ± 0.82	2.14 ± 1.10	7.04 ± 0

All enzymatic activities are expressed in U/g wet weight of muscle tissue. (1 U = 1 μmol substrate metabolized in 1 minute). *N.d.* not determined. * Cytochrome oxidase activity in the presence of 80 μM cytochrome c; ** Cytochrome oxidase activity in the presence of 200 μM cytochrome c (as described in the Methods section)

(complex I), cytochrome c oxidase (complex IV) and succinate:cytochrome c reductase (complex II + III) in skeletal muscle homogenates. Absolute enzymatic activities are shown in Table 3. In conformity with the enhanced mitochondrial staining in some of the biopsy specimens of PD patients we observed a marked increase of CS activity (PD patients: 17.1 ± 4.2 U/mg wet weight, controls 12.6 ± 4.5 U/mg wet weight, $p < 0.0005$). The one patient who gave report to perform regular physical training (No. 7) exhibited the highest CS activity. The absolute enzymatic activities of the complexes I + III, II + III and IV correlated tightly with the CS activity ($p < 0.005$ for all enzymes) with the exception of the complex IV activity measured with 200 μM cytochrome c, where one data point (patient 1) was aberrantly low.

To consider differences in mitochondrial quantity in the individual muscle samples, the activities have to be normalized with respect to the mitochondrial marker enzyme citrate synthase (CS). As shown in Table 4, a significant decrease of the mean activities of the CS-normalized complexes I, I + III, and IV (with 200 μM cytochrome c) were detectable in PD muscle. A slight but not significant decrease was also present in the complex II + III activity of PD patients. It was not possible to find any differences using the ''classical'' complex IV assay (with 80 μM cytochrome c).

Table 4. Citrate synthase-normalized enzymatic activities in homogenates and respiration rates plus inhibitor titrations of saponin-permeabilized skeletal muscle fibers

	Controls (N = 36)	PD patients	Level of significance
NADH: cytochrome c reductase/ citrate synthase	0.23 ± 0.11	0.16 ± 0.05 (N = 19)	$p < 0.01$
NADH: Coenzyme Q1 reductase/ citrate synthase	0.12 ± 0.04	0.08 ± 0.03 (N = 16)	$p < 0.01$
succinate: cytochrome c reductase/ citrate synthase	0.15 ± 0.05	0.13 ± 0.03 (N = 19)	n.s.
cytochrome c oxidase/citrate synthase (80 μM cytochrome c)	0.18 ± 0.08	0.18 ± 0.06 (N = 19)	n.s.
cytochrome c oxidase/citrate synthase (200 μM cytochrome c)	0.57 ± 0.20	0.40 ± 0.16 (N = 13)	$p < 0.05$
pyruvate + malate respiration of permeabilized muscle fibers	8.5 ± 2.9	9.54 ± 2.4 (N = 19)	n.s.
glutamate + malate respiration of permeabilized muscle fibers	7.3 ± 3.3	9.9 ± 2.2 (N = 19)	$p < 0.005$
succinate respiration of permeabilized muscle fibers	11.5 ± 4.2	12.8 ± 2.8 (N = 19)	n.s.
flux control coefficient of complex I (amytal titration)	0.22 ± 0.06	0.31 ± 0.09 (N = 19)	$p < 0.001$
flux control coefficient of complex IV (azide titration)	0.24 ± 0.05	0.29 ± 0.07 (N = 19)	$p < 0.005$

The respiration rates are determined at 25°C and expressed in nmol O2/min/mg dry weight. The flux control coefficients are determined by inhibitor titrations of the maximal respiration (in the presence of 2 mM ADP) with the substrates glutamate + malate for complex I and glutamate + malate + succinate for complex IV. *N.s.* not significant

Biochemistry in permeabilized muscle fibers

To investigate the mitochondrial function in PD muscle in more detail, we decided to use the ''permeabilized muscle fibers'' technique. These muscle fibers, when treated with low concentrations of saponin, have been shown to be a suitable model to study oxidative phosphorylation in skeletal muscle under conditions much more similar to the *in vivo* situation than isolated mitochondria or skeletal muscle homogenates (Kunz et al., 1993). The maximal rates of respiration of these fibers are shown in Table 4. In agreement with the elevated CS activity in PD muscle, the ADP-stimulated respiration rates of saponin-permeabilized fibers of PD patients are higher than in control preparations.

To evaluate the metabolic effect of the relative complex I and IV deficiencies, we applied inhibitor titrations with the specific noncompetitive inhibitors of complex I – amytal – and complex IV – sodium azide (Hattori et al., 1991). Typical amytal titrations of the ADP-stimulated muscle fiber respiration rate from a control subject are shown in Fig. 1A. ADP addition initially causes maximal stimulation of the pyruvate + malate oxidation rate. Since glutamate is able to further increase the respiration rate in the presence of 1 mM pyruvate and 5 mM malate, we added 10 mM glutamate to obtain the maximal oxygen consumption rate that can be reached with complex I-dependent substrates. Subsequent

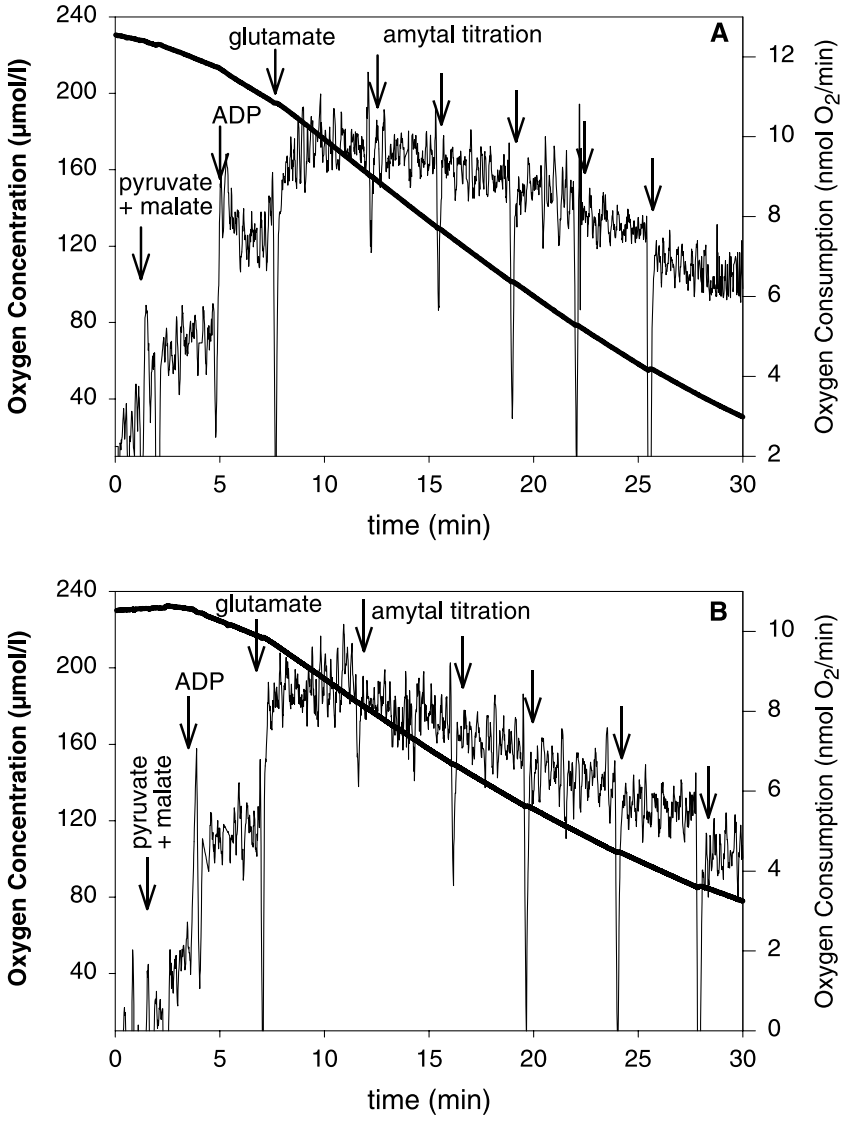

Fig. 1. Amytal titration of respiration in saponin-permeabilized skeletal muscle fibers. **A** Control patient, **B** patient with PD. *Bold line*: Oxygen concentration in the measuring chamber. *Fine line*: Oxygen consumption of permeabilized muscle fibers as derivate of first order of the oxygen concentration

amytal additions caused a decrease of the respiration rate due to complex I inhibition. A similar experiment performed with skeletal muscle fibers from a patient with PD is shown in Fig. 1B. Again, ADP stimulated the fiber respiration, and glutamate addition led to the maximal respiration rate. In contrast to the controls, amytal caused a more pronounced initial inhibition of the rate of muscle fiber respiration. This result can be explained by possible differences in the amytal sensitivity of complex I or by a deficiency of the entire enzyme. To obtain a quantitative value for the change in amytal sensitivity of muscle fiber respiration, we calculated flux control coefficients of complex I which allows a

quantitative description of control exerted by this enzyme within the framework of metabolic control analysis (a value of 1 is equivalent to maximal control; a value of 0 is equivalent to no control (Kacser and Burns, 1973; Heinrich and Rapoport, 1974)). A summary of these values for all patients is given in Table 4. The quantitative data show that the complex I flux control coefficient in PD muscle fibers is elevated. Similar investigations were performed with the respiratory chain complex IV inhibitor azide. Again, a higher azide sensitivity of respiration was observed in most PD muscle samples. The quantitative expression of this higher azide sensitivity is the increased flux control coefficient for complex IV in PD skeletal muscle fibers (Table 4). These results are in line with the decreased CS normalized activities of complexes I and IV in the muscle homogenate.

Sequencing and Southern blot analysis

Since point mutations in the tRNA-cluster coding for the tRNAs Ile, Glu and Met and in the adjacent ND2 gene of mitochondrial respiratory complex I had been reported in patients with Parkinson's disease (Shoffner et al., 1993), we screened these two small regions by PCR and automated sequencing. In summary we could detect 27 variations from the Cambridge sequence (Anderson et al., 1981). All of these polymorphisms were previously reported as non-pathogenic in the MITOMAP database (MITOMAP, 2003), or they did not alter the amino acid sequence. We could not detect any heteroplasmic point mutation within the level of detection of automated sequencing which is above 10% degree of heteroplasmy. All sequence variants were equally found in mtDNA extracted from blood and from skeletal muscle specimens. We also screened mtDNA in PD skeletal muscle for large-scale rearrangements (i.e. duplications and deletions) and for quantitative changes (so-called ''mtDNA depletion''), using Southern blot techniques. We could not identify rearrangements of the mtDNA in PD muscle within the level of detection. This confirms the observations of other authors (DiDonato et al., 1993; Shoffner et al., 1991). Comparison of the relative amount of mtDNA to nuclear DNA did reveal a slight increase of mtDNA in PD skeletal muscle, confirming the observed increase of mitochondrial mass (data not shown).

Quantification of point mutated mtDNA by DPD analysis

Total DNA isolated from skeletal muscle samples from 19 patients with PD and from 9 controls was subjected to DPD to detect mtDNA point mutations (pm-mtDNA) in two small regions of the mtDNA. The DPD experiments were repeated twice, starting from the DNA extraction step. In Figs. 2 and 3, we show sets of experiments that were representative of our results. In panel A (in both figures) we present original data printouts, generated by the GeneScan® analysis software after the first PCR and digestion step, and after electrophoretic separation on a capillary sequencer. It should be noted that traces of uncleaved DNA, if existing, are far below the level of detection. After the second round of PCR and digestion we checked the quality and quantity of PCR fragments on silver-stained polyacrylamide gels (panel B, Figs. 2 and 3).

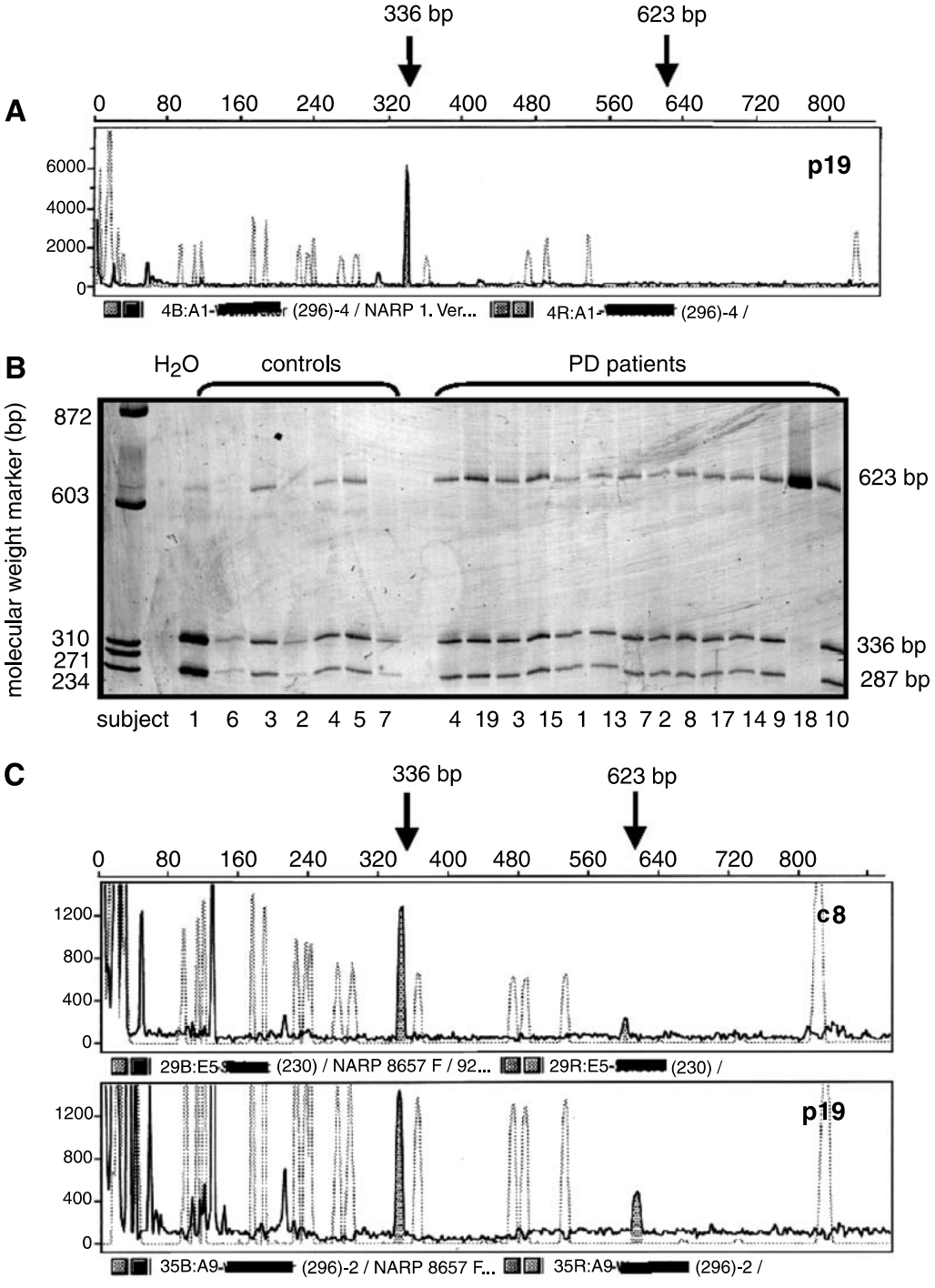

Fig. 2. Double PCR and digestion of a representative skeletal muscle mtDNA fragment containing the restriction site nt 8991–8995. **A** Original GeneScan® analysis printout of one PCR fragment (obtained from PD patient p19) after the first digestion step. *Dotted line*: ROX fragment size standard. *Full line*: subject's PCR product. No peak is detectable at bp 623. **B** Silver-stained polyacrylamide gel with PCR products after the second round of PCR and digestion. **C** GeneScan® analysis printouts of one representative control patient (c8) and one representative PD patient (c19). The 6-FAM labeled digested PCR products are visible as peaks of 623 bp (for the uncleaved PCR fragment, indicative for pm-mtDNA) and 336 bp (for the cleaved fragment, i.e. wild type mtDNA)

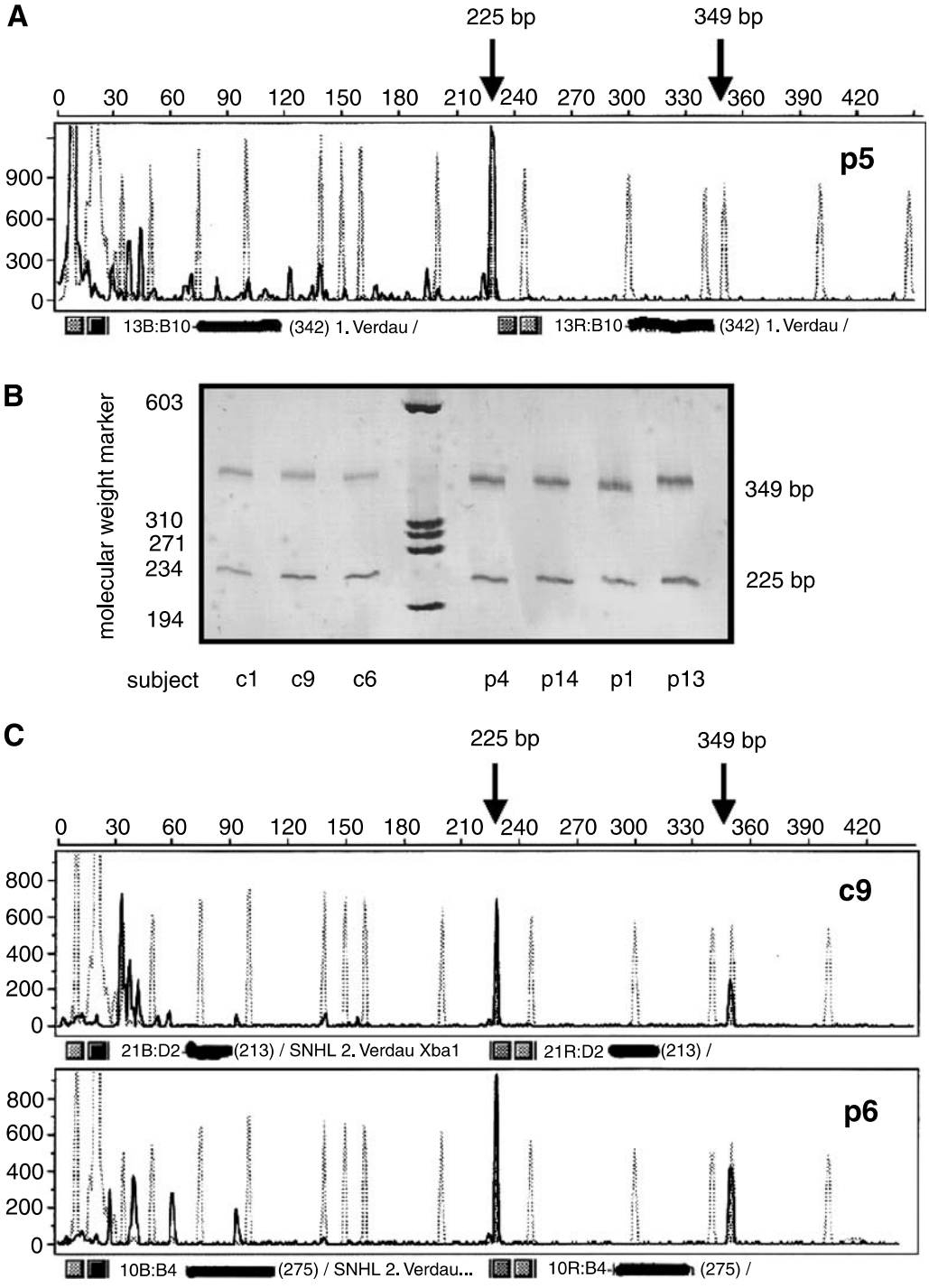

Fig. 3. Double PCR and digestion of a representative skeletal muscle mtDNA fragment containing the restriction site nt 7440–7445. **A** Original GeneScan® analysis printout of one PCR fragment (obtained from PD patient p5) after the first digestion step. *Dotted line*: ROX fragment size standard. *Full line*: subject's PCR product. No peak is detectable at 349 bp. **B** Silver-stained polyacrylamide gel with PCR products after the second round of PCR and digestion. **C** GeneScan® analysis printouts of one representative control patient (c9) and one representative PD patient (c6). The 6-FAM labeled digested PCR products are visible as peaks of 349 bp (for the uncleaved PCR fragment, indicative for pm-mtDNA) and 225 bp (for the cleaved fragment, i.e. wild type mtDNA)

Because a quantification of the stained DNA on these gels seemed to be not accurate enough, we performed electrophoretic separation on the capillary sequencer again and used the GeneScan® analysis software to calculate the relative amount of uncleaved (i.e., mutated) DNA with respect to the cleaved (i.e., wild type) DNA by determination of the peak areas of the two peaks representing the 6-FAM labelled PCR fragments (Figs. 2 and 3, panel C).

In the nt 8991–8995 region (Fig. 2), we detected pm-mtDNA in six of the controls and in all of the PD patients. The peak of uncleaved DNA (623 bp) was below the level of detection (as defined by the baseline of the fluorescence signal in GeneScan® analysis) in 3 single experiments in control samples. The highest ratio of pm-mtDNA in controls was $23.1 \pm 2.5\%$. As shown in Fig. 4, we found a statistically significant difference between the average ratios of the pm-mtDNA in controls ($11.2 \pm 10.5\%$) and in PD-patients ($23.6 \pm 4.5\%$; $p < 0.001$). One sample (No. 18, see Fig. 2B) was not cleavable by *MvaI*, leading to apparently 100% ''mutated'' DNA at the particular site. The sample was sequenced to clarify the problem, and we found a presumably homoplasmic base pair change (G8994A) without changing of the amino acid (leucine) but leading to a change in the recognition sequence of the restriction endonuclease. The sample was therefore excluded from further statistical analysis. The highest ratio of pm-mtDNA was $31.3 \pm 0.70\%$ in PD patient No. 19.

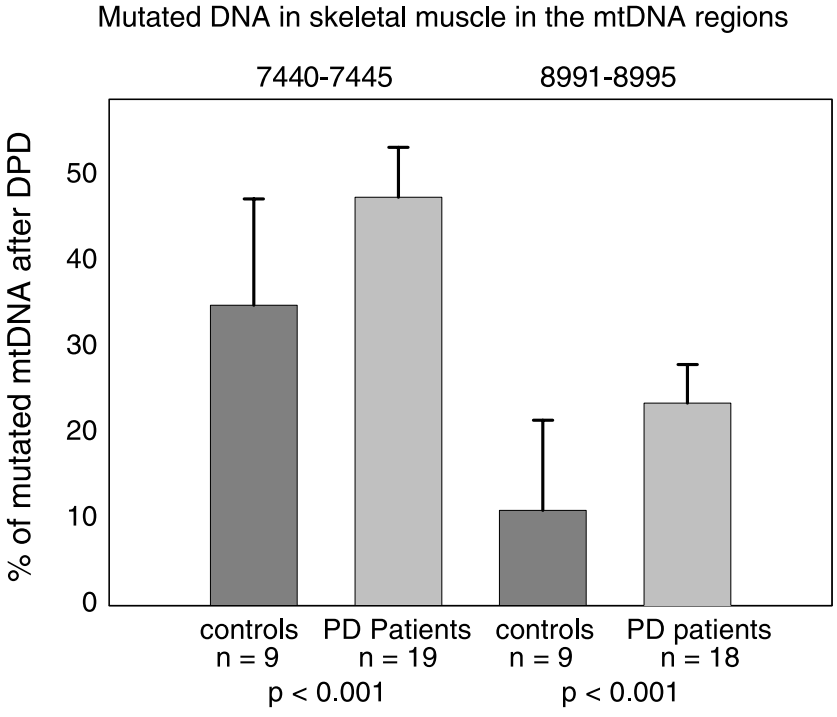

Fig. 4. Relative amount of mtDNA harbouring point mutations in the mtDNA regions nt 7440–7445 (the *XbaI* recognition sequence) and nt 8991–8995 (the *MvaI* recognition sequence) in skeletal muscle of PD patients and controls. The amount of pm-mtDNA is expressed in % of total amount of the amplified mtDNA fragment. *N* number of subjects

Mutations in the nt 7440–7445 region (Fig. 3) were detectable in all of the samples. The highest ratio of pm-mtDNA in controls was $47.3 \pm 0.4\%$. The highest ratio of pm-mtDNA was found in PD patient No. 4 ($60.3 \pm 8.9\%$). We found a statistically significant difference between the average ratios of the pm-mtDNA in controls ($35.0 \pm 12.4\%$) and in PD-patients ($47.6 \pm 5.8\%$; $p < 0.001$, see Fig. 4).

Since each DNA sample was isolated from muscle tissue samples containing several hundred muscle fibers, it was not clear if the rise of point mutations was restricted to a subset of fibers, or if pm-mtDNA was present in virtually all muscle fibers. Thus, we subjected DNA which was isolated from small bundles of dissected muscle fibers (usually used for respiration experiments) containing 2–4 single fibers (checked by microscopy) to the DPD procedure. An amount of 10 fiber bundles for each of the subjects 6, 12, and two controls was taken for the investigation. The percentage of point mutations in ''oligo-fiber'' bundles was nearly the same as in mtDNA from the large tissue samples of the same patient, suggesting that mtDNA point mutations were almost equally distributed all over the skeletal muscle rather than clonal expanded in certain single muscle fibers (data not shown).

We could not find a statistically significant correlation between L-Dopa medication (Table 2), amount of point-mutated mtDNA, UPDRS, and biochemical mitochondrial function in skeletal muscle. This observation is a strong argument that the increase of pm-mtDNA as well as the mitochondrial impairment is not closely related to L-Dopa medication.

Discussion

The mitochondrial involvement in PD cannot as yet be assigned (Orth and Schapira, 2001), but there is conclusive evidence that mitochondrial dysfunction in PD is related to an imbalance between generation and detoxification of reactive oxygen species (Koutsilieri et al., 2002). Many authors share the conviction that the mitochondrial dysfunction in PD is restricted to complex I of the respiratory chain, in particular in the substantia nigra. However, in our opinion there is no convincing reason to assure that a disturbance of free radical metabolism in PD is present exclusively in the brain. Data about respiratory chain enzymes in PD in extracerebral tissues like skeletal muscle are highly controversal (see Table 1). In agreement with others (Singer et al., 1995; Orth and Schapira, 2002), we believe that this inconsistency is most likely due to methodological problems. In this study, we have tried to minimize factors which are possibly responsible for misleading results in the analysis of mitochondrial enzymes: (i) underestimation of difficulties in enzymatic assays; (ii) the selection of suitable control subjects; and (iii) the question whether to prefer isolated skeletal muscle mitochondria or skeletal muscle homogenate for enzymatic assays.

The spectrophotometric analysis of skeletal muscle homogenate is rather problematic because of a heterogenous portion of lipids and connective tissue and because of the turbidity of the sample. However, we repudiate that there is advantage to analyze isolated mitochondria because of possible loss of the

presumably more fragile (i.e., swollen) defective mitochondria, resulting in an artificial enrichment of non-affected (i.e., ''normal'') mitochondria in such preparations. Thus, we and others have developed techniques for highly reliable analysis of mitochondrial function in skeletal muscle homogenates (Miro et al., 1998; Trounce et al., 1996; Wiedemann et al., 2000) and permeabilized cells and muscle fibers for the analysis of mitochondria in their *in situ* environment (Kuznetsov et al., 1998; Sperl et al., 1997). Using this optimized methods, we confirmed the presence of a combined defect of complexes I and IV in PD muscle (Shoffner et al., 1991; Bindoff et al., 1991; Cardellach et al., 1993; Blin et al., 1994). The activity of the NADH: succinate dehydrogenase (complex II + III) was not significantly decreased. However, it is likely that a mixed enzymatic activity with low absolute values like the succinate: cytochrome c reductase assay render the freedom from damage of complexes II and/or III uncertain (Singer et al., 1995). Other authors have shown that there could be indeed a deficiency of complex II or II + III in PD skeletal muscle (see Table 1). To confirm our results of a mild deficiency of NADH:CoQ oxidoreductase and cytochrome c oxidase in skeletal muscle of PD patients, we applied two technically independent methods-high resolution respirometry and metabolic control analysis- to saponin-permeabilized muscle fibers. We would like to emphasize our point of view (Vielhaber et al., 2003) that the biochemical investigation of mitochondrial function should be not based on one single method.

One surprising observation is the abundance of mitochondria and CS activity in most of the PD samples despite the partial immobilisation of many PD patients. It might be of interest to find out if there is a correlation between rigor, tremor, and mitochondrial proliferation. Moreover, it could be of importance if immobilisation and muscular disuse might affect the protein content of specific respiratory chain complexes. However, the latter seems unlikely to us since absolute activities of COX and succinate: cytochrome c reductase were higher in PD muscle than controls.

It is likely that a generalized defect of respiratory chain complexes is due to direct oxidative damage on proteins (Koutsilieri et al., 2002). However, it needs to be clarified whether there is also a genetic basis of the observed respiratory chain dysfunction. It was recently hypothesized that certain mtDNA sequence variations could be associated to higher accumulation of ROS (van der Walt et al., 2003). On the other hand, it is clear that PD is not a classical mitochondrial disorder characterized by point mutations or rearrangements of the mtDNA (Byrne, 2002). It is surprising that all previous attempts to reveal mtDNA mutations in PD were focused on either mtDNA deletions or on *single* mtDNA point mutations at a relatively *high level of heteroplasmy* (i.e., at least 5% of total mtDNA). Despite the recognition that mtDNA repair is robust (Mason et al., 2003; Bohr et al., 2002), the hypothesis that free radicals damage the mtDNA is largely conclusive: There are hints that base excision repair in mitochondria is not as effective as in nuclear DNA (Saxowsky et al., 2002; Grishko et al., 1999; Karahalil et al., 2002) and declines with age (Chen et al., 2002). Thus, it seemed very likely that free radicals induce multiple, randomly distributed, somatic point mutations of the mtDNA, presumably at extremely low levels

of heteroplasmy, in all regions of the mitochondrial genome. We therefore decided to address the question as to whether pm-mtDNA is present in low levels of heteroplasmy in skeletal muscle of PD patients. In previous studies on aging individuals, it was shown by PCR/RFLP analysis that the level of heteroplasmy of pm-mtDNA was approximately 0.1% of total mtDNA (Zhang et al., 1993). Because of these low levels, it could not be excluded that incomplete digested DNA or DNA polymerase misincorporation could lead to inaccurate results. In order to avoid this problem, we used the DPD method (Pallotti et al., 1996; Wiedemann et al., 2002). Compared with other PCR-based methods (i.e., real time PCR), DPD has the advantage that it detects any point mutation in the endonuclease recognition sequence. Furthermore, the method is not very prone to produce artifacts. It was shown by other authors that phenol in DNA extraction solutions rarely increases the measurable level of pm-mtDNA (Helbock et al., 1998). In contrast, any preparation-caused DNA fragmentation could easily imitate multiple mtDNA deletions.

We report here a significant increase of pm-mtDNA in two regions of the mtDNA circle in skeletal muscle. This increase is about 1.5 to 2-fold to the pm-mtDNA ratio in control subjects. However, DPD only estimates the proportion of pm-mtDNA in a *short stretch* of the genome (i.e. the endonuclease recognition sequence: 5–6 bp). If we assume that potentially any mtDNA position could be mutated at low levels, and that some of these somatic mutations could be pathogenic, it is possible to envision a situation where a large number of mtDNAs in every cell are defective. Another point of concern is that the abnormal amount of pm-mtDNA might be due to aging. However, other authors provide evidence that specific mtDNA point mutations may not accumulate in skeletal muscle during normal human aging (Pallotti et al., 1996).

The fact that the relative amount of point mutated mtDNA in ''oligo-fiber bundles'' was equal to that from whole muscle tissue samples indicates that some degree of mtDNA damage is present in virtually all fibers simultaneously. It is therefore very likely that the elevation of pm-mtDNA in PD compared to controls is the result of a generalized free radical-induced DNA damage. Free-radical induced point mutations could be rapidly induced but also rapidly removed by mtDNA repair. Thus, the observed point mutations can hardly accumulate to a higher degree of heteroplasmy. On the other hand, if these mutations are present during transcription and translation, the biosynthesis of respiratory chain complexes could be impaired.

A point of critizism could be the fact that we could not find a statistically significant correlation between the ratio of pm-mtDNA and the biochemical data. This is, however, not surprising, because it is likely that there are other mtDNA regions within the same individual containing much more or less mtDNA point mutations. To our knowledge there is no other method to test the overall load of *low-heteroplasmic* point mutations in mtDNA. It was previously shown that the absolute amounts of mutated mtDNA after DPD were far below 1% of total mtDNA in each mtDNA region screened (Pallotti et al., 1996; Wiedemann et al., 2002). Here we did not quantify the absolute amounts of point-mutated DNA by subjecting the relative amounts to a standard curve

again, because we have had the experience that this is a technically demanding task without giving new information.

In summary, our data support the presence of a mild but generalized mitochondrial defect in patients with PD which is accompanied by an increase of point mutated mtDNA in skeletal muscle. In consideration of the presumed adaption of mitochondrial mass it is unlikely that the observed slight deficiencies of complex I and IV have noticeable effects on the muscular strength of PD patients. However, our observation underlines that pathogenetic factors of neurodegeneration are not completely restricted to certain regions of the brain. A complex mechanism, involving free radical production and/or detoxification, specific enhancement of free radical damage in some neuronal structures, and impairment of mtDNA repair could be responsible for the etiopathogenesis of PD.

Acknowledgements

We thank the volunteer Parkinson patients who donated tissue for this investigation. The generous support by grant from the "Deutsche Parkinson-Vereinigung e.V. (Neuss) and the excellent technical assistance of K. Kaiser, I. Schellhase, and J. Witzke are gratefully acknowledged. The work was supported by grant BMBF NBL3 (01220107).

References

Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290: 457–465

Anderson JJ, Bravi D, Ferrari R, Davis TL, Baronti F, Chase TN, Dagani F (1993) No evidence for altered muscle mitochondrial function in Parkinson's disease. J Neurol Neurosurg Psychiatry 56: 477–480

Bergmeier HU (1974) Citratsyntase. In: Bergmeier HU (ed) Methoden der enzymatischen Analyse. Wissenschaftsverlag, Weinheim, pp 727–733

Bindoff LA, Birch-Machin M, Cartlidge NE, Parker WD Jr, Turnbull DM (1989) Mitochondrial function in Parkinson's disease. Lancet 2: 49

Bindoff LA, Birch-Machin MA, Cartlidge NE, Parker WD Jr, Turnbull DM (1991) Respiratory chain abnormalities in skeletal muscle from patients with Parkinson's disease. J Neurol Sci 104: 203–208

Blin O, Desnuelle C, Rascol O, Borg M, Peyro Saint PH, Azulay JP, Bille F, Figarella D, Coulom F, Pellissier JF (1994) Mitochondrial respiratory failure in skeletal muscle from patients with Parkinson's disease and multiple system atrophy. J Neurol Sci 125: 95–101

Bohr VA, Stevnsner T, Souza-Pinto NC (2002) Mitochondrial DNA repair of oxidative damage in mammalian cells. Gene 286: 127–134

Byrne E (2002) Does mitochondrial respiratory chain dysfunction have a role in common neurodegenerative disorders? J Clin Neurosci 9: 497–501

Cardellach F, Marti MJ, Fernandez-Sola J, Marin C, Hoek JB, Tolosa E, Urbano-Marquez A (1993) Mitochondrial respiratory chain activity in skeletal muscle from patients with Parkinson's disease. Neurology 43: 2258–2262

Chen D, Cao G, Hastings T, Feng Y, Pei W, O'Horo C, Chen J (2002) Age-dependent decline of DNA repair activity for oxidative lesions in rat brain mitochondria. J Neurochem 81: 1273–1284

DiDonato S, Zeviani M, Giovannini P, Savarese N, Rimoldi M, Mariotti C, Girotti F, Caraceni T (1993) Respiratory chain and mitochondrial DNA in muscle and brain in Parkinson's disease patients. Neurology 43: 2262–2268

Di Monte DA, Sandy MS, Jewell SA, Langston JW (1993) Oxidative phosphorylation by intact muscle mitochondria in Parkinson's disease. Neurodegeneration 2: 275–281

Dubowitz V, Brooke MH (1973) Muscle biopsy: a modern approach. Saunders, Philadelphia
Gasser T (2001) Genetics of Parkinson's disease. J Neurol 248: 833–840
Grishko VI, Druzhyna N, LeDoux SP, Wilson GL (1999) Nitric oxide-induced damage to mtDNA and its subsequent repair. Nucl Acids Res 27: 4510–4516
Hattori N, Tanaka M, Ozawa T, Mizuno Y (1991) Immunohistochemical studies on complexes I, II, III, and IV of mitochondria in Parkinson's disease. Ann Neurol 30: 563–571
Heinrich R, Rapoport TA (1974) A linear steady-state treatment of enzymatic chains. Critique of the crossover theorem and a general procedure to identify interaction sites with an effector. Eur J Biochem 42: 97–105
Helbock HJ, Beckman KB, Shigenaga MK, Walter PB, Woodall AA, Yeo HC, Ames BN (1998) DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. Proc Natl Acad Sci USA 95: 288–293
Holt IJ, Harding AE, Petty RK, Morgan-Hughes JA (1990) A new mitochondrial disease associated with mitochondrial DNA heteroplasmy. Am J Hum Genet 46: 428–433
Jenner P (2003) Oxidative stress in Parkinson's disease. Ann Neurol 53 [Suppl 3]: S26–S36
Kacser H, Burns JA (1973) Rate control of biological processes. Cambridge University Press, London
Karahalil B, Hogue BA, Souza-Pinto NC, Bohr VA (2002) Base excision repair capacity in mitochondria and nuclei: tissue-specific variations. FASEB J 16: 1895–1902
Kirches EJ, Winkler K, Warich-Kirches M, Szibor R, Wien F, Kunz WS, von Bossanyi P, Bajaj PK, Dietzmann K (1998) mtDNA depletion and impairment of mitochondrial function in a case of a multisystem disorder including severe myopathy. J Inherit Metab Dis 21: 400–408
Koutsilieri E, Scheller C, Grunblatt E, Nara K, Li J, Riederer P (2002) Free radicals in Parkinson's disease. J Neurol 249 [Suppl 2]: II1–II5
Kunz WS, Kuznetsov AV, Schulze W, Eichhorn K, Schild L, Striggow F, Bohnensack R, Neuhof S, Grasshoff H, Neumann HW (1993) Functional characterization of mitochondrial oxidative phosphorylation in saponin-skinned human muscle fibers. Biochim Biophys Acta 1144: 46–53
Kuznetsov AV, Winkler K, Kirches E, Lins H, Feistner H, Kunz WS (1997) Application of inhibitor titrations for the detection of oxidative phosphorylation defects in saponin-skinned muscle fibers of patients with mitochondrial diseases. Biochim Biophys Acta 1360: 142–150
Kuznetsov AV, Mayboroda O, Kunz D, Winkler K, Schubert W, Kunz WS (1998) Functional imaging of mitochondria in saponin-permeabilized mice muscle fibers. J Cell Biol 140: 1091–1099
Mann VM, Cooper JM, Krige D, Daniel SE, Schapira AH, Marsden CD (1992) Brain, skeletal muscle and platelet homogenate mitochondrial function in Parkinson's disease. Brain 115(Pt 2): 333–342
Mason PA, Matheson EC, Hall AG, Lightowlers RN (2003) Mismatch repair activity in mammalian mitochondria. Nucl Acids Res 31: 1052–1058
Miro O, Cardellach F, Barrientos A, Casademont J, Rotig A, Rustin P (1998) Cytochrome c oxidase assay in minute amounts of human skeletal muscle using single wavelength spectrophotometers. J Neurosci Methods 80: 107–111
MITOMAP (2004) A human mitochondrial genome database. http://www.mitomap.org
Mizuno Y, Ohta S, Tanaka M, Takamiya S, Suzuki K, Sato T, Oya H, Ozawa T, Kagawa Y (1989) Deficiencies in complex I subunits of the respiratory chain in Parkinson's disease. Biochem Biophys Res Commun 163: 1450–1455
Nakagawa-Hattori Y, Yoshino H, Kondo T, Mizuno Y, Horai S (1992) Is Parkinson's disease a mitochondrial disorder? J Neurol Sci 107: 29–33
Orth M, Schapira AH (2001) Mitochondria and degenerative disorders. Am J Med Genet 106: 27–36
Orth M, Schapira AH (2002) Mitochondrial involvement in Parkinson's disease. Neurochem Int 40: 533–541
Pallotti F, Chen X, Bonilla E, Schon EA (1996) Evidence that specific mtDNA point mutations may not accumulate in skeletal muscle during normal human aging. Am J Hum Genet 59: 591–602

Reichmann H, Janetzky B, Bischof F, Seibel P, Schols L, Kuhn W, Przuntek H (1994) Unaltered respiratory chain enzyme activity and mitochondrial DNA in skeletal muscle from patients with idiopathic Parkinson's syndrome. Eur Neurol 34: 263–267

Reid FM, Vernham GA, Jacobs HT (1994) A novel mitochondrial point mutation in a maternal pedigree with sensorineural deafness. Hum Mutat 3: 243–247

Richter G, Sonnenschein A, Grunewald T, Reichmann H, Janetzky B (2002) Novel mitochondrial DNA mutations in Parkinson's disease. J Neural Transm 109: 721–729

Saks VA, Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T, Tranqui L, Olivares J, Winkler K, Wiedemann F, Kunz WS (1998) Permeabilized cell and skinned fiber techniques in studies of mitochondrial function in vivo. Mol Cell Biochem 184: 81–100

Saxowsky TT, Matsumoto Y, Englund PT (2002) The mitochondrial DNA polymerase beta from Crithidia fasciculata has 5′-deoxyribose phosphate (dRP) lyase activity but is deficient in the release of dRP. J Biol Chem 277: 37201–37206

Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD (1989) Mitochondrial complex I deficiency in Parkinson's disease. Lancet 1: 1269

Sciacco M, Bonilla E (1996) Cytochemistry and immunocytochemistry of mitochondria in tissue sections. Meth Enzymol 264: 509–521

Shoffner JM, Watts RL, Juncos JL, Torroni A, Wallace DC (1991) Mitochondrial oxidative phosphorylation defects in Parkinson's disease. Ann Neurol 30: 332–339

Shoffner JM, Brown MD, Torroni A, Lott MT, Cabell MF, Mirra SS, Beal MF, Yang CC, Gearing M, Salvo R (1993) Mitochondrial DNA variants observed in Alzheimer disease and Parkinson disease patients. Genomics 17: 171–184

Singer TP, Ramsay RR, Ackrell BA (1995) Deficiencies of NADH and succinate dehydrogenases in degenerative diseases and myopathies. Biochim Biophys Acta 1271: 211–219

Sperl W, Skladal D, Gnaiger E, Wyss M, Mayr U, Hager J, Gellerich FN (1997) High resolution respirometry of permeabilized skeletal muscle fibers in the diagnosis of neuromuscular disorders. Mol Cell Biochem 174: 71–78

Taylor DJ, Krige D, Barnes PR, Kemp GJ, Carroll MT, Mann VM, Cooper JM, Marsden CD, Schapira AH (1994) A 31P magnetic resonance spectroscopy study of mitochondrial function in skeletal muscle of patients with Parkinson's disease. J Neurol Sci 125: 77–81

Trounce IA, Kim YL, Jun AS, Wallace DC (1996) Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines. Meth Enzymol 264: 484–509

van der Walt JM, Nicodemus KK, Martin ER, Scott WK, Nance MA, Watts RL, Hubble JP, Haines JL, Koller WC, Lyons K, Pahwa R, Stern MB, Colcher A, Hiner BC, Jankovic J, Ondo WG, Allen FH Jr, Goetz CG, Small GW, Mastaglia F, Stajich JM, McLaurin AC, Middleton LT, Scott BL, Schmechel DE, Pericak-Vance MA, Vance JM (2003) Mitochondrial polymorphisms significantly reduce the risk of Parkinson disease. Am J Hum Genet 72: 804–811

Van Kuilenburg AB, Dekker HL, Van den BC, Nieboer P, Van Gelder BF, Muijsers AO (1991) Isoforms of human cytochrome-c oxidase. Subunit composition and steady-state kinetic properties. Eur J Biochem 199: 615–622

Vielhaber S, Kudin A, Winkler K, Wiedemann F, Schroder R, Feistner H, Heinze HJ, Elger CE, Kunz WS (2003) Is there mitochondrial dysfunction in amyotrophic lateral sclerosis skeletal muscle? Ann Neurol 53: 686–687

Wharton DC, Tzagoloff A (1967) Cytochrome oxidase from beef heart mitochondria. Meth Enzymol 10: 245–250

Wiedemann FR, Winkler K, Kuznetsov AV, Bartels C, Vielhaber S, Feistner H, Kunz WS (1998) Impairment of mitochondrial function in skeletal muscle of patients with amyotrophic lateral sclerosis. J Neurol Sci 156: 65–72

Wiedemann FR, Winkler K, Lins H, Wallesch CW, Kunz WS (1999) Detection of respiratory chain defects in cultivated skin fibroblasts and skeletal muscle of patients with Parkinson's disease. Ann NY Acad Sci 893: 426–429

Wiedemann FR, Vielhaber S, Schroder R, Elger CE, Kunz WS (2000) Evaluation of methods for the determination of mitochondrial respiratory chain enzyme activities in human skeletal muscle samples. Anal Biochem 279: 55–60

Wiedemann FR, Manfredi G, Mawrin C, Beal MF, Schon EA (2002) Mitochondrial DNA and respiratory chain function in spinal cords of ALS patients. J Neurochem 80: 616–625

Zhang C, Linnane AW, Nagley P (1993) Occurrence of a particular base substitution (3243 A to G) in mitochondrial DNA of tissues of ageing humans. Biochem Biophys Res Commun 195: 1104–1110

Authors' address: Dr. F. R. Wiedemann, Klinik für Neurologie, Universitätsklinikum Magdeburg, Leipziger Strasse 44, D-39120 Magdeburg, Germany, e-mail: falk.wiedemann@medizin.uni-magdeburg.de