Bioenergetic and proteolytic defects in fibroblasts from patients with sporadic Parkinson's disease

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ABSTRACT

Background: Parkinson's disease (PD) is a complex disease and the current interest and focus of scientific research is both investigating the variety of causes that underlie PD pathogenesis, and identifying reliable biomarkers to diagnose and monitor the progression of pathology. Investigation on pathogenic mechanisms in peripheral cells, such as fibroblasts derived from patients with sporadic PD and age/gender matched controls, might generate deeper understanding of the deficits affecting dopaminergic neurons and, possibly, new tools applicable to clinical practice.
Methods: Primary fibroblast cultures were established from skin biopsies. Increased susceptibility to the PD-related toxin rotenone was determined with apoptosis- and necrosis-specific cell death assays. Protein quality control was evaluated assessing the efficiency of the Ubiquitin Proteasome System (UPS) and protein levels of autophagic markers. Changes in cellular bioenergetics were monitored by measuring oxygen consumption and glycolysis-dependent medium acidification. The oxido-reductive status was determined by detecting mitochondrial superoxide production and oxidation levels in proteins and lipids.
Results: PD fibroblasts showed higher vulnerability to necrotic cell death induced by complex I inhibitor rotenone, reduced UPS function and decreased maximal and rotenone-sensitive mitochondrial respiration. No changes in autophagy and redox markers were detected.
Conclusions: Our study shows that increased susceptibility to rotenone and the presence of proteolytic and bioenergetic deficits that typically sustain the neurodegenerative process of PD can be detected in fibroblasts from idiopathic PD patients. Fibroblasts might therefore represent a powerful and minimally invasive tool to investigate PD pathogenic mechanisms, which might translate into considerable advances in clinical management of the disease.

1. Introduction

A number of converging, still incompletely characterized factors contribute to the pathogenesis of Parkinson's disease (PD). Age, predisposing genetic background and exposure to environmental stressors may cooperate in triggering the disease by affecting neuronal mechanisms responsible for cellular homeostasis, such as mitochondrial

function and protein quality control [1,2]. Mitochondrial dysfunctions have been repeatedly shown in PD patients and experimental PD models [3] and the resulting alterations in cellular redox and energy homeostasis might be crucial to PD pathobiology [4]. Decreased efficiency of the Ubiquitin Proteasome System (UPS) and autophagic pathways – two major cellular systems of protein quality control – is also likely to contribute to PD pathogenesis [5]; the presence of aggregated, poly-ubiquitinated proteins in Lewy Bodies (LBs) clearly indicates that proteolytic dysfunction and proteo-toxicity are critical steps in the pathogenic cascade of PD [6]. Direct evidence of these pathobiological processes can be obtained only in postmortem brains; it is therefore essential to identify and develop peripheral markers of biomolecular dysfunctions relevant to PD that might have a substantial impact in the clinical setting. Such biomarkers could be used to monitor disease

progression, efficacy of new therapies, as well as to investigate whether the complexity of phenotypes shown by PD patients is paralleled by heterogeneity of biomolecular responses. Numerous studies have been conducted in peripheral cells of PD patients, mostly isolated from venous blood (i.e. lymphomonocytes, platelets, red blood cells), on the assumption that a generalized biomolecular defect associated with a predisposing genetic background could be detectable in any cell of the body. Results have been often contradictory and, in most cases, the influence of pharmacological treatments (levodopa, in particular) has represented a strong confounding factor [7]. Several studies have been recently relying on fibroblasts, as a substrate to investigate PD mechanisms [8]. Fibroblasts represent an easily accessible source of proliferating cells that share the same genetic complexity of neurons [9]; cultured fibroblasts have also the advantage, compared to peripheral blood cells, of being exposed to the effects of anti-PD drugs at much lower concentrations as compared to peripheral blood cells, since they need to be grown and replicated in culture for several days before being used for the experiments. The aim of this study was, therefore, to use primary fibroblasts derived from patients with sporadic PD and healthy controls, to investigate whether and how the pathogenic mechanisms knowingly associated with neurodegeneration in PD can be detected in these peripheral cells. In particular, we focused on the exacerbated susceptibility to pro-oxidant drugs, the impaired function of quality control systems, especially the machinery involved in protein degradation, and mitochondrial defects, both in terms of redox control and respiratory capacity [1]. The more general and ambitious objective was to identify markers of PD-related pathobiology that might be used to develop predictive models for the identification of PD patients within the elder population.

2. Subjects and methods

2.1. Cell culture

Fibroblasts were obtained from sporadic PD patients and age- and sex-matched healthy controls (Table 1). The research was approved by the Ethic Committee of the National Neurological Institute "C. Mondino" of Pavia, and informed consent was obtained from all subjects. All fibroblast strains were isolated from skin biopsies of the upper medial arm and cultured under highly standardized conditions in RPMI 1640-AQmedia (Sigma) complemented with 1% Streptomycin and Penicillin antibiotics and 20% Fetal Bovine Serum (FBS, Sigma). Cells used in the experiments were grown in adhesion and expanded in flasks up-to a maximum of 13 passages. Indeed, the number of passages can affect cell senescence and impact on biomolecular responses. Therefore the number of passages in vitro for each fibroblast cell line used in the experiments was controlled and kept consistent within groups to avoid biases associated with cell replication [10]. Furthermore, fibroblasts were used for the experiments only when the cell culture had reached at least 80% confluency, with the only exception of the respirometry experiments in the Seahorse XF Extracellular Flux Analyzer, in which cells were confluent (see Section 2.6).

Table 1
Clinical data of subjects involved in the study.

	Controls	PD patients
N (M/F)	7 (3/4)	11 (6/5)
Age (years)	62.8 ± 4.8	64.1 ± 5.3
Disease duration (years)	–	12.1 ± 6
UPDRS (subscale III) score	–	19.8 ± 5.3
Hoehn and Yahr stage	–	2.3 ± 0.7
L-dopa daily intake (mg)	–	738.9 ± 362.3

All patients were undergoing treatment with levodopa. Five out of eleven were taking dopamine agonists as adjunctive therapy. UPDRS-III score (motor subscale) and Hoehn and Yahr stage were evaluated in the "ON" phase. Values are expressed as mean ± sd.

In some experiments fibroblasts were treated with PD-associated neurotoxin rotenone (Sigma). Rotenone was dissolved in DMSO and a 100 mM stock was prepared. Rotenone was subsequently diluted directly in the medium to the final concentrations of 500 and 20 μM and cells were exposed to the toxin for 6 h for the evaluation of protein quality control system and cell viability/apoptosis. Lower concentrations (100 nM, 1 μM and 10 μM) and shorter time of exposure (1 h) were adopted for the redox experiments.

2.2. Autophagic markers, poly-ubiquitination and macromolecule oxidation

Protein lysates were obtained by resuspending fibroblast pellets in ice-cold lysis buffer (CelLytic, Sigma) containing sodium fluoride (1:100, Sigma) and diluted Phosphatase (1:10, Roche) and Protease inhibitors (1:25, Roche). After centrifugation, the supernatant was collected and protein concentration was measured using a Bicinchoninic Acid (BCA) Protein Assay (Sigma).

For poly-ubiquitinated proteins, crude cell lysates were obtained by resuspending fibroblast pellets in SDS-containing loading buffer (Tris–HCl 0.5 M pH 6.8, 10% SDS, 25% Glycerol, 0.5% bromophenol blue and 5% β-mercaptoethanol in milliQ water).

All of the protein lysates were run on 4–12% gels (Invitrogen) or 10% gels (Biorad), transferred onto nitrocellulose membranes (Invitrogen) and western blot was performed. Membranes were blocked (Odyssey blocking buffer, LiCor) and incubated overnight with primary antibodies (actin (sc-1616, 1:8000), ubiquitin (sc-166553, 1:1000) and LAMP2 (sc-5571, 1:2000)), Santa Cruz; mTOR (#4517, 1:1000), phospho-mTOR (#2971, 1:500) and parkin (#2132, 1:500), Cell Signaling; beclin1 (3663-100, 1:500), BioVision; LC3 (L7543, 1:500) and p62/SQSTS1 (P0067, 1:2000), Sigma; 4-Hydroxynonenal or 4-HNE (ab46545, 1:500), phospho-Ser129 α-synuclein (ab59264, 1:500), α-synuclein (ab51252, 1:1000), and Abcam and secondary antibodies (IRDye® 700 goat anti-mouse, IRDye® 800 goat anti-rabbit (1:10,000), LiCor, Biosciences). Image analysis was performed using the fluorescent near-infrared Odyssey®scanner and software (LiCor, Biosciences) and fluorescence was normalized with the corresponding Actin signal.

Protein carbonyls were measured following derivatization with 2,4-Dinitrophenylhydrazine (DNPH). Briefly, samples were prepared in H_2O_2 containing 12% SDS. Positive samples were incubated 15 min with a solution containing 20 mM DNPH in 5% TFA and the reaction was stopped by adding 6.7 μl of neutralization solution (2 M Trizma base containing 30% Glycerol and 2-mercaptoethanol). Samples were loaded onto 4–12% precast gel (Invitrogen), transferred onto PVDF membranes (Millipore) after SDS electrophoresis, finally 2,4-Dinitrophenylhydrazone (DNP)-derivates were detected after incubation with anti-dinitrophenyl (DNP) primary antibody (D8406, 1:1000, Sigma) and a rat anti-mouse IgE-HRP secondary antibody (1130-05, 1:1000, SouthernBiotech) diluted in PBS + 5% milk. Blots were developed by chemiluminescence. Negative controls were prepared by avoiding DNPH addition. To normalize the DNP signal, amine reactive carboxylic-acid succimidyl (CAS) ester conjugated to Alexa680 (1:100, Invitrogen) was added to each sample and analyzed using the fluorescent near-infrared Odyssey®scanner and software. The chemiluminescent signal was detected using an Alliance 2.7 scanning system. Images were quantified using Metamorph software.

2.3. 20S proteasome and caspase 3 activity

Activities of 20S proteasome and caspase 3 were assessed on fibroblast lysates with ELISA commercial kits (Enzo Life Science and Molecular Probes). The 20S proteasome is the catalytic subunit of the UPS and is therefore marker of proteolytic activity/homeostasis. Caspase 3 is one of the major downstream factors involved in the apoptotic cascade and is therefore marker of programmed cell death. Briefly, fibroblast pellets were resuspended in ice-cold PBS, lysed by freezing–thawing cycles and centrifuged. Protein-containing supernatants were

loaded together with standards, positive and negative controls in 96-well plates and incubated with a fluorogenic substrate (Suc-LLVY-AMC and Z-DEV-R110, for detecting respectively the 20S proteasome and caspase 3 activity), following the manufacturer's instructions. Results were expressed as a measure of fluorescence produced after enzymatic cleavage and were detected with a microplate reader (Molecular Devices). Values of fluorescence were normalized with the protein content in each sample.

2.4. Cell viability and apoptosis

Cell viability and apoptosis were evaluated by staining fibroblasts with SYTOX Green nucleic acid stain (Invitrogen) and Annexin V (BD Biosciences) dyes. SYTOX Green is a dye which specifically binds nucleic acids in cells and is able to access DNA only when cell membranes are disrupted and cells are dead. SYTOX Green is therefore marker of necrotic cell death. Differently, Annexin V is a dye which selectively binds phosphatidylserine, a phospholipid typically localized on the inner side of the plasma membrane. During the initial phases of apoptosis, phosphatidylserine residues are flipped on the outer side of the membrane and can be detected by the dye. Annexin V is therefore marker of early apoptosis. Cells were treated for 6 h with rotenone (20 and 500 μM), washed in $1\times$ Annexin V binding Buffer (0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM CaCl$_2$, diluted 1:10) and incubated in the dark with 0.5 μM SYTOX Green and 5 μl Annexin V, following manufacturer instructions. Negative controls were also included. After 15 min, 400 μl $1\times$ Annexin V binding Buffer was added and fluorescence was detected for each sample using a FACS analyzer (FASCanto-BD Biosciences). Measures were taken in duplicates and normalized by subtracting the blank. Analysis was performed with FlowJo software (TreeStar).

2.5. Evaluation of cellular thiol redox status and mitochondrial superoxide production

Before performing these experiments, fibroblasts were exposed to rotenone as described in Section 2.1. The redox immunocytochemistry is a double-labeling technique, in which two different fluorophore-conjugated dyes are used to detect reduced and oxidized cysteines in tissues and cell preparations [11,12]. The assay consisted in 3 steps: cells were fixed, permeabilized and thiols (SH) were labeled with 2 μM AlexaFluor555-maleimide dye (Invitrogen) in the presence of 1 mM N-ethylmaleimide (NEM, Sigma); disulfides (SS) were reduced by the addition of 5 mM tris(2-carboxyethyl)phosphine (TCEP, Sigma) and were finally labeled by the addition of 2 μM AlexaFluor488-maleimide (Invitrogen) in the presence of 1 mM NEM. Images were taken in both AlexaFluor555 and AlexaFluor488 emission channels using a confocal microscope; the redox state (SS/SH) was calculated as the ratio between the images in the two channels. Analysis was subsequently carried out using MetaMorph software (Olympus).

Mitochondrial superoxide production was detected by staining cells with MitoSOX dye (Invitrogen). Fibroblasts were incubated in the dark at 37 °C for 20 min with 2 μM MitoSOX diluted in HBSS (Hanks balanced salt solution, Gibco), according to the cell type and following manufacturer instructions. Negative controls were also included. At the end of the incubation, cells were centrifuged and resuspended in 500 μl of FACS resuspending buffer (PBS, 0.2% FBS and 0.02% Sodium Azide). Fluorescence was immediately detected for each sample using a FACS analyzer (FASCanto-BD Biosciences). Measures were taken in duplicates and normalized by subtracting the blank. Analysis was performed with FlowJo software (TreeStar).

2.6. Mitochondrial respiration and glycolysis

The Seahorse XF Extracellular Flux Analyzer enables to measure simultaneously and in real-time mitochondrial respiration and glycolysis

in cultured cells. Respiration and glycolysis are respectively measured as the rate of oxygen consumption (OCR) and extracellular acidification (ECAR); the latter is principally caused by conversion of pyruvate to lactate and therefore provides an estimate of the glycolytic flux. The system also allows to inject up-to four drugs during the experimental run and to monitor their effects over time. According to the standard protocol, we injected in the following order (final concentrations are indicated): 1 μM oligomycin (ATP-synthase inhibitor); 0.4 μM carbonyl, cyanide-4-(trifluoromethoxy) phenylhydrazone (or FCCP, oxidative phosphorylation uncoupler); 0.5 μM rotenone (complex I inhibitor) and 1 μM antimycin A (complex III inhibitor). Cells were plated the day before the experiment and optimal cell density (60000 cells/well) was determined experimentally to ensure a proportional response to FCCP with cell number (data not shown). This density resulted in confluent cultures, in which cell growth was blocked due to contact inhibition, thereby avoiding potential biases due to different growth rates in PD and control fibroblasts. Absence of cell replication was verified in a parallel experiment, in which control fibroblasts were seeded at the same density used in the Seahorse experiment and counted after 16 h in culture, using a Beckman Coulter Z2 Cell and Particle Counter. No cell replication was detected in this timeframe. Moreover, we observed no differences in mitochondrial morphology, density and distribution, after staining control and PD-derived fibroblasts with the potentiometric dye CMXRos MitoTracker Red (Invitrogen), which labels functional mitochondria with active membrane potential. MitoTracker quantification was performed in an unbiased fashion, using the MetaMorph software (Olympus), to quantify emission intensity and total area (i.e. mitochondrial polarization levels and area of polarized mitochondria per cell). The analysis did not show any statistical difference between PD and control cells (data not shown).

Five measurements were taken for both OCR and ECAR at baseline and four measurements after each toxin injection. The cumulative OCR and the ECAR were calculated at baseline and over time after each drug injection. The values of both OCR and ECAR at any time point were calculated as the average of multiple measurements for the same cell line (n = 5). No cell death was observed throughout the experiment.

Eight bioenergetic parameters were also calculated from the analysis of the OCR values, according to previously published reports [13,14]. Basal respiration was measured as the average of OCR values at baseline, before toxin injections. Non-mitochondrial respiration is the final OCR measure after antimycin A. Values for each one of the remaining parameters were calculated as the difference of OCR measures after and before injection of specific toxins. Respiration for ATP-synthesis: OCR (basal-oligomycin); proton leakage: OCR (oligomycin–antimycin A); maximum respiration: OCR (FCCP–antimycin A); rotenone-sensitive respiration (mainly complex I-driven): OCR (FCCP–rotenone); rotenone-insensitive respiration: OCR (rotenone–antimycin A); and reserve capacity (FCCP-basal).

2.7. ATP levels

ATPlite 1 step (PerkinElmer) is a luminescence assay that monitors ATP levels in living cells. Fibroblasts were plated in a 96-well plate (CulturPlate, PerkinElmer) and luminescence was measured in a microplate reader after the addition of ATP-dependent enzyme luciferase and its substrate luciferin, following manufacturer's instructions. A standard curve was prepared to calculate the ATP values of the samples. Final values were normalized with the protein content in each sample.

2.8. Statistical analysis

Statistical analysis was performed with Prism5 (GraphPad software). Unpaired t-test was applied and 95% confidence interval was set for statistical significance. Results were considered significant with p-value < 0.05.

3. Results

3.1. Cell viability and apoptosis

Cell viability and early apoptosis were evaluated at baseline and after incubation with 20 and 500 μM of rotenone. Rotenone is a mitochondrial complex I inhibitor which interferes with oxidative phosphorylation by depleting energy production, augmenting ROS formation and eventually causing cell death. At the highest dose, rotenone induced necrotic cell death in both PD patients and controls. This effect, however, was significantly enhanced in PD fibroblasts (Fig. 1A). The percentage of apoptotic cells did not change after rotenone administration and no differences were observed between PD patients and control fibroblasts. In untreated cells, there were no differences in necrosis or apoptosis among the groups, as also indicated by unchanged basal caspase 3 activity (Fig. 1B and C).

3.2. Proteolytic mechanisms part I: 20S proteasome and UPS

Basal activity of 20S proteasome, the catalytic subunit of the UPS, was significantly reduced in PD as compared to control fibroblasts. Higher accumulation of ubiquitinated proteins, representative of

Fig. 1. Cell viability and apoptosis in control and PD fibroblasts. Cell death assays after (A) SYTOX Green and (B) Annexin V staining at baseline (vehicle) and after rotenone administration (20 and 500 μM), and (C) measure of basal caspase 3 activity (controls: n = 6, PD patients: n = 11). Results are expressed as mean ± sem. $p < 0.05 vs. vehicle, *p < 0.05 vs. controls.

impaired 26S proteasome function, and increased levels of parkin protein, encoded by PD-associated gene PARK2, were found in PD as compared to control cells at baseline (Fig. 2A, C and D). In order to investigate the mechanisms underlying the increase in necrosis after rotenone administration, we measured possible changes in the activity of 20S proteasome and in the expression of autophagic markers (see Section 3.3.). We observed no significant changes in 20S proteasome activity after rotenone exposure as compared to baseline in either control or PD fibroblasts. However, we observed significantly higher induction (expressed as % baseline-vehicle) of 20S proteasome activity in PD fibroblasts as compared to controls after both 20 and 500 μM rotenone administration (Fig. 2B).

We also checked for α-synuclein and phosphorylated-Ser129 α-synuclein protein levels, but they were far too low for validation by western blotting (data not shown).

3.3. Proteolytic mechanisms part II: autophagy

We analyzed basal levels of autophagy modulators such as phosphorylated-mTOR and beclin1, LAMP2, a marker associated to chaperone-mediated autophagy, as well as LC3-II/LC3-I ratio and p62, representative of autophagic vesicle accumulation. None of these markers were significantly different between groups at baseline (Fig. 3).

After treating cells with rotenone (20 and 500 μM), we could not detect any significant change in protein levels for any of these markers as compared to baseline, except from increased LC3-II accumulation in both groups after exposure to 500 μM rotenone (Fig. 3A). When comparing control and PD fibroblasts, most of the changes in the expression of autophagic markers were detectable after administration of 20 μM rotenone. At this concentration, p62, mTOR phosphorylation and LAMP2 were down-regulated, but not significantly different between groups (Fig. 3B–D–E). Only beclin1 protein levels were significantly lower in PD cells (Fig. 3C). Finally, no significant differences were detected in the induction (expressed as % baseline-vehicle) of autophagic marker expression between PD and controls, with the exception of LAMP2 (Supplementary Fig. 1).

3.4. Mitochondrial bioenergetics and redox homeostasis

The bioenergetic properties of PD and control fibroblasts were assessed using the Extracellular Flux Analyzer from Seahorse Bioscience (Fig. 4). According to the OCR values, eight bioenergetic parameters were also calculated and significant decrease was found in maximal respiration and rotenone-sensitive respiration (Table 2). Basal ATP levels were unchanged between groups (Supplementary Fig. 2) consistent with the lack of changes in respiration dedicated to ATP-synthesis. We also investigated the glycolytic capacity in our fibroblast lines by measuring extracellular acidification rate (ECAR) due to augmented proton production, which is in turn caused by increased conversion of pyruvate to lactate (i.e. glycolysis). We measured basal and stimulated ECAR. In the latter condition, mitochondrial function and ATP production are perturbed by oligomycin, FCCP, or rotenone and glycolytic flux increases to compensate (Fig. 4). Both basal and stimulated ECAR did not differ between PD fibroblasts and respective controls (Fig. 4B).

Basal production of mitochondrial superoxide and cellular redox status (measured as the ratio between disulfides and reduced thiols) were unchanged in PD versus control fibroblasts. Also, no significant changes and differences could be observed in protein thiol oxidation when fibroblasts were exposed to rotenone within a range of concentrations similar to the one (0.5 μM) adopted to challenge complex I in the bioenergetic experiments (Fig. 5A to C). Finally, no differences could be detected in the accumulation of irreversibly oxidized macromolecules, such as protein carbonyls and lipid peroxidation markers at baseline (Fig. 5D to G).
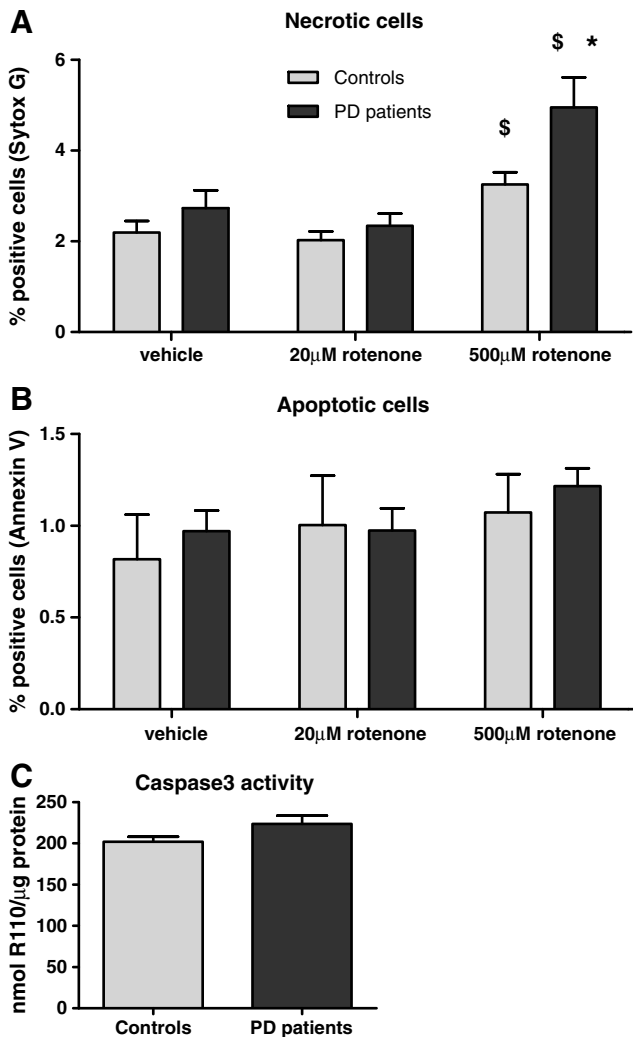
A

Proteasome 20S activity

B

rotenone and 20S proteasome activity

C

poly-ubiquitinated proteins
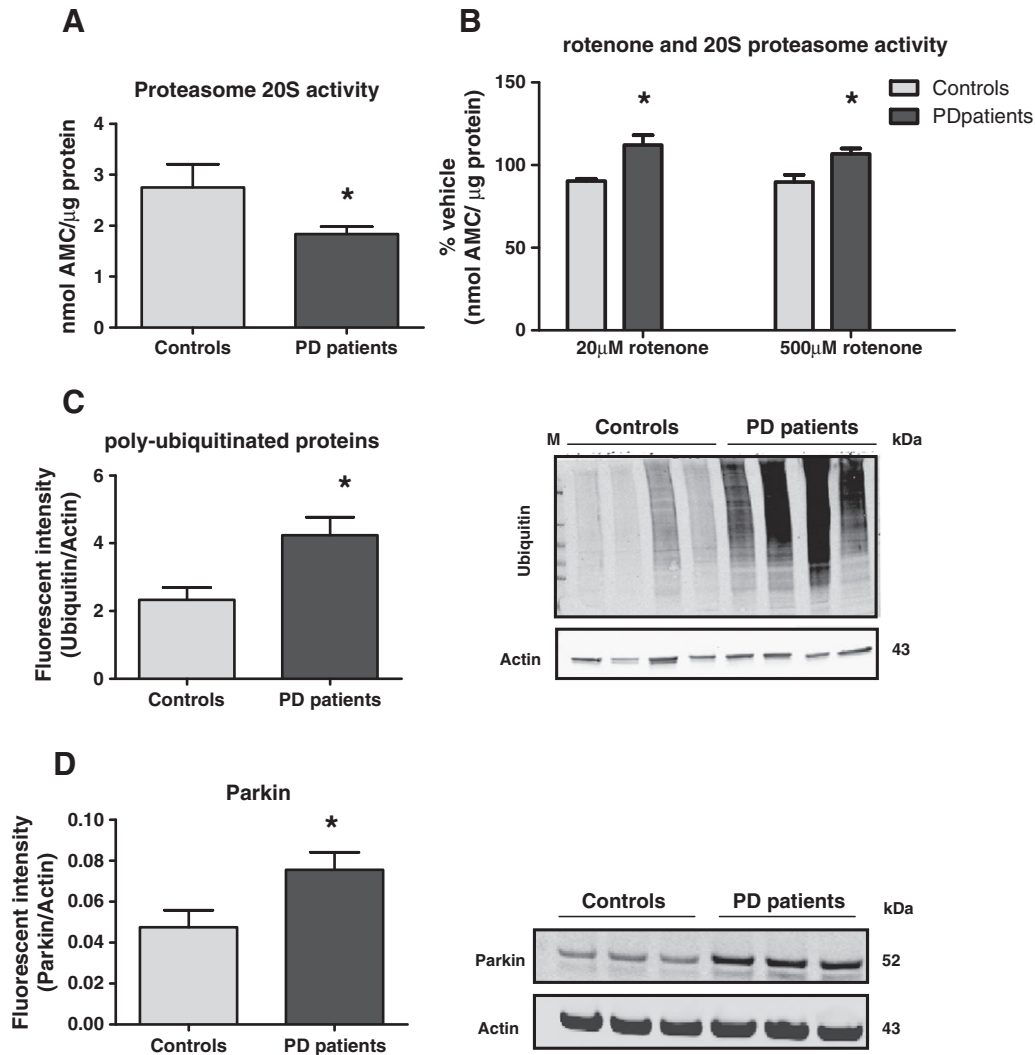

D

Parkin

Fig. 2. UPS function and parkin protein levels in control and PD fibroblasts. Measure of (A) 20S proteasome activity at basal level and (B) change in the activity of the 20S proteasome after exposure to rotenone (20 and 500 μM for 6 h) as compared to baseline (vehicle), (C) poly-ubiquitinated proteins, representative of UPS function, and (D) fluorescence measured for E3-ubiquitin ligase, parkin. Right: representative membranes blotted with anti-ubiquitin and anti-parkin antibodies; (controls: n = 7, PD patients: n = 11). Results are expressed as mean ± sem; *p < 0.05 vs. controls.

4. Discussion

In this study we compared cell viability, redox homeostasis, protein quality control and cellular bioenergetics in fibroblasts derived from patients with sporadic PD and controls.

According to recent publications, the identification of diagnostic and prognostic biomarkers of PD needs to fulfill essential, scientifically shared criteria. For instance, candidate biomarkers should be representative of disease status and/or progression and most importantly should be linked to fundamental features and mechanisms underlying PD neuropathology [7,15,16]. Also, the use of skin-derived cells to investigate brain diseases with the aim of identifying biomarkers and potentially new treatments is attracting increasing interest [17–19]. Several studies have been performed on fibroblasts derived from genetic and sporadic patients with neurodegenerative diseases, in particular Alzheimer's disease [20–22], Huntington's disease [23] and, very recently, also genetic forms of Amyotrophic Lateral Sclerosis [24–26]. All these studies have used fibroblasts as a model to investigate the mechanisms underlying the pathology with the idea of describing how peripheral cells express the molecular and metabolic defects that are typical of the degenerating neurons. Many papers have also been published using fibroblasts as a model to investigate PD pathogenesis. Most of these published data focus on specific pathological features and use cells from patients with genetic PD, carrying PINK1, parkin or LRRK2 mutations [27–30]. In this study we used fibroblasts obtained from patients with idiopathic PD, in which the etiopathogenesis is due to contribution of a complex genetic background rather than monogenic causes. We analyzed a broad panel of markers to identify significant differences between PD and control fibroblasts and possibly to test the predictive potential of this cell type in the investigation on PD pathobiology.

The main achievement of this study is that we could detect, in skin fibroblasts, some deficits associated with PD pathogenesis that are typically reported at central level.

We demonstrated a critical impairment in the activity of the 20S proteasome, the catalytic subunit of the UPS and significantly higher accumulation of poly-ubiquitinated proteins, indicating that the proteolytic function of the UPS is affected in PD fibroblasts as well as in the brain. Previous data from our lab showed impaired 20S proteasome activity also in lymphocytes derived from patients with sporadic PD [31], confirming that alterations in UPS activity are generally detectable in peripheral cells. Differently, we could not detect differences in the basal levels of autophagic markers between PD and control fibroblasts. Our results are in contrast with previously published data showing that autophagic marker expression is increased in peripheral blood cells derived from PD patients as compared to controls [32,33]. However, the mismatch might be attributed to the differences between the two
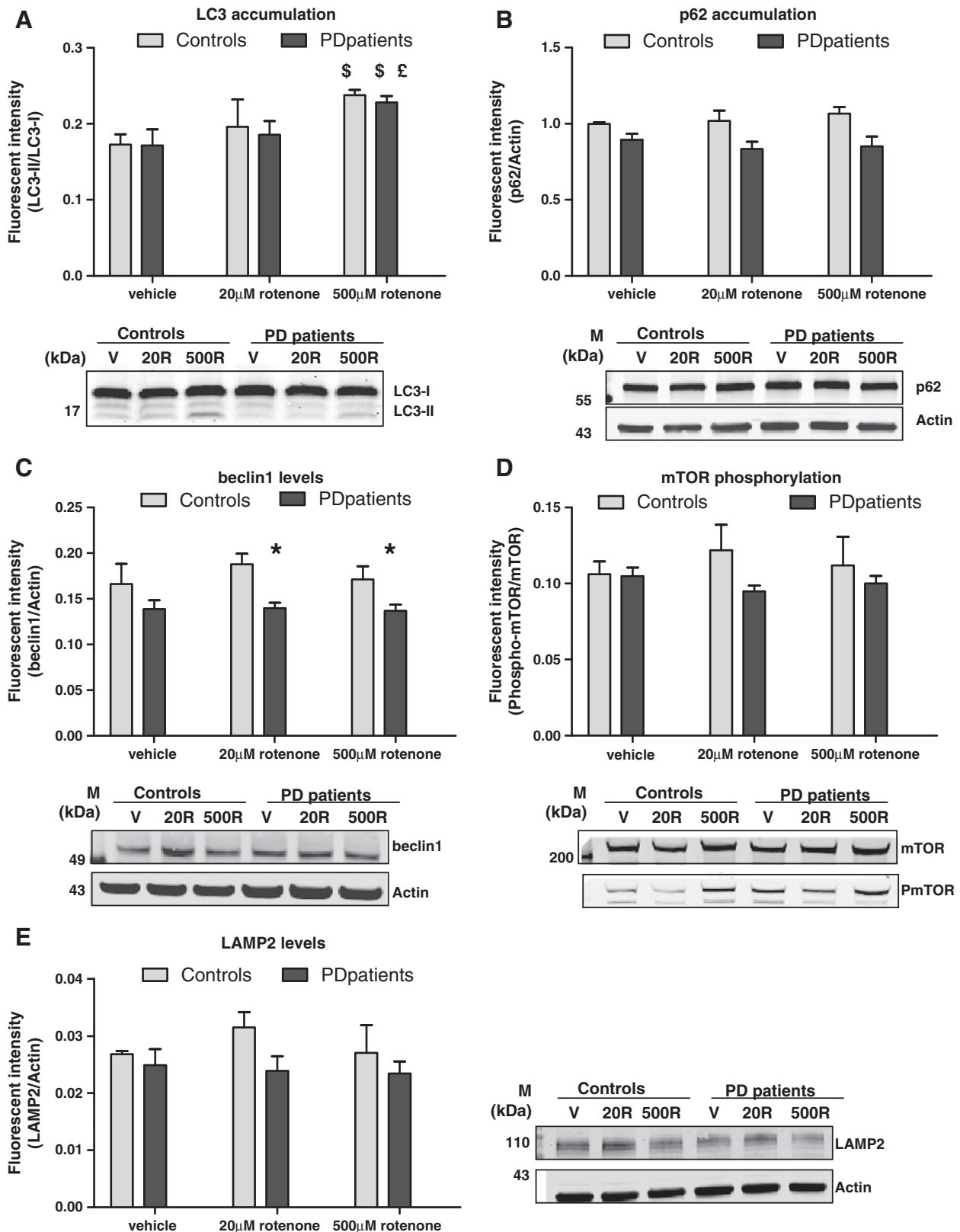
A LC3 accumulation

B p62 accumulation

C beclin1 levels

D mTOR phosphorylation

E LAMP2 levels

Fig. 3. Autophagic markers expression in control and PD fibroblasts. Protein levels of autophagy-associated markers (controls: n = 6, PD patients: n = 10): (A) LC3-II/LC3-I; (B) p62; (C) beclin1; and (D) phosphorylated mTOR/total mTOR and (E) LAMP2 at baseline (vehicle) and after 6-hour rotenone (20 and 500 μM) exposure. Below or side: representative membranes obtained after western blotting for all the above-mentioned markers and treatment conditions (V: vehicle, 20R: 20 μM rotenone, 500R: 500 μM rotenone). Results are expressed as mean ± sem; *p < 0.05 vs. controls, $p < 0.05 vs. vehicle, £p < 0.05 vs. 20 μM rotenone.

cellular systems (fibroblasts versus blood cells) and the effect of levodopa treatment on leukocytes. We found that parkin protein levels are higher in fibroblasts from sporadic PD patients. Parkin is a cytoplasmic

E3 ubiquitin ligase, which catalyzes ubiquitination of target proteins. Mutations in the PARK2 gene lead to loss of function of parkin protein and therefore accumulation of non-degraded proteins. Also, parkin is
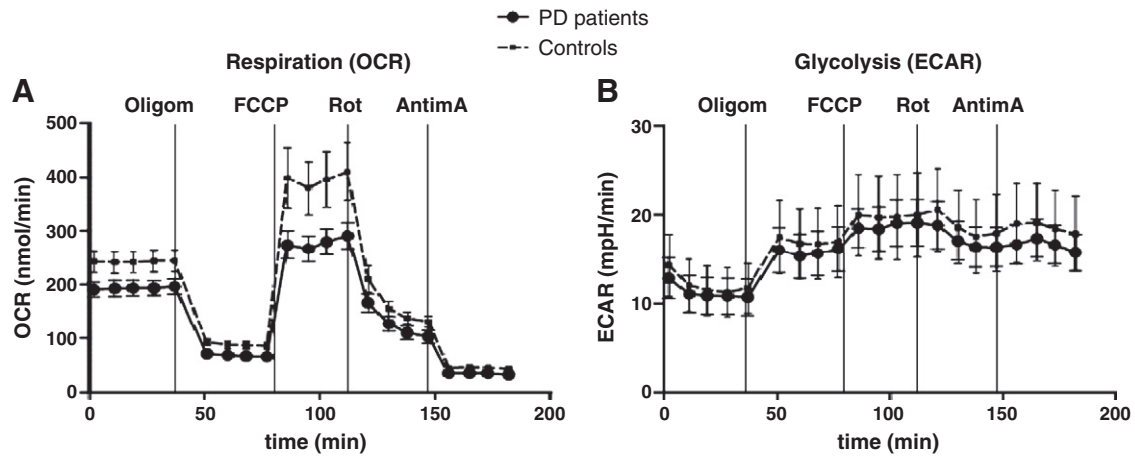
Fig. 4. Mitochondrial respiration and glycolysis in control and PD fibroblasts. Measures obtained with the Extracellular Flux Analyzer for (A) OCR representative of mitochondrial respiration and (B) ECAR representative of glycolysis. Dotted and full lines represent controls and PD patients respectively (controls: n = 7, PD patients: n = 11). Oligom: Oligomycin; FCCP: Carbonyl, cyanide-4-(trifluoromethoxy)phenylhydrazone; Rot: Rotenone; AntimA: Antimycin A.

involved in signaling and maintenance of mitochondria, especially by sustaining ATP synthesis and promoting mitophagy [34–36]. In our study, increased levels of parkin might be associated with the impairment of UPS function in two different ways. The UPS is responsible for parkin degradation and increased levels of the protein might be a consequence of such deficit. Alternatively, parkin might be over-expressed in order to compensate for UPS impaired function [37], for its own loss of function [38] and possibly, in line with recent evidence, to sustain the bioenergetic requirement of cells [39]. Indeed in our study, basal respiration and respiration dedicated to ATP synthesis were lower, but not significantly different between PD and control fibroblasts, in line with the observation that no changes in basal ATP levels could be detected.

We found that protein levels for both non-phosphorylated and phosphorylated-Ser129 α-synuclein were below detection capacity, as also shown by others [40]. In their study, Hoepken and collaborators showed that α-synuclein mRNA, but not protein levels is detectable in fibroblasts from PINK1 gene (PARK6) and sporadic PD patients. Interestingly, they found that α-synuclein expression was consistently increased in patients carrying mutations in PINK1-encoding gene, but not in all the sporadic ones, indicating that in fibroblasts from idiopathic PD patients mRNA levels for α-synuclein are highly variable.

Similarly, we could not observe differences in any of the markers reflecting intracellular redox state, despite it was previously found in fibroblasts carrying mutations in PINK1 that redox state is altered already at basal level [41]. However, Del Hoyo and collaborators [42], who analyzed the levels of antioxidant defenses such as coenzyme Q10 and the activity of superoxide-dismutase, glutathione peroxidase and catalase in fibroblasts from sporadic patients, found no differences in the comparison with the control samples, thereby supporting our findings. We hypothesize that other compensatory mechanisms scavenging

Table 2
Bioenergetic parameters (nmol/min).

	Controls	PD patients	p-value
Basal respiration	198.90 ± 51.89	160.53 ± 37.72	0.09
Respiration for ATP-synthesis	154.25 ± 41.23	125.32 ± 33.21	0.12
Proton leakage	44.66 ± 14.26	35.05 ± 8.95	0.09
Maximum respiration	352.22 ± 136.03	244.74 ± 68.31	0.04[*]
Rotenone-sensitive respiration	264.68 ± 114.84	174.74 ± 48.71	0.03[*]
Rotenone-insensitive respiration	87.54 ± 27.01	70.01 ± 27.21	0.19
Reserve capacity	153.31 ± 100.44	84.36 ± 54.70	0.07
Non-mitochondrial respiration	43.19 ± 10.64	32.63 ± 17.81	0.18

Bioenergetic parameters obtained from OCR values measured on PD patient and control fibroblasts. Maximum respiration and rotenone-sensitive respiration were significantly lower in PD fibroblasts as compared to controls. Results are expressed as mean ± sem.
  [*] p < 0.05 vs. controls.

ROS and protecting cell viability are active already at baseline and significant changes on cell viability and oxidative stress tolerance in PD fibroblasts might be observed only after critical challenge and perturbations occur, as already suggested by others [43]. In line with this affirmation, our data offer further support to the hypothesis that PD cells respond differently to stressing conditions and activate a different pattern of compensatory mechanisms to counteract perturbations. In PD fibroblasts, we demonstrated increased vulnerability to the administration of complex I inhibitor rotenone, in agreement with previous findings from Cooper and collaborators, who showed that iPSC-derived neural cells are susceptible to the same drugs that affect viability in the fibroblast strains from which they were reprogrammed [27]. Interestingly, they highlighted that fibroblasts respond to the same pro-oxidants, proteasome inhibitors and mitochondrial stressors, but at much higher concentrations as compared to iPSC-derived neural cells, thereby supporting our observation that PD fibroblasts show differences in viability only after exposure to high doses of rotenone.

Rotenone is a complex I inhibitor and can also interfere with autophagy and UPS activity [44]. Although in PD fibroblasts the UPS is already inhibited at baseline, these cells tend to activate the proteasome rather than autophagy in response to rotenone, as represented by higher induction of 20S proteasome activity and lower levels of autophagic marker expression. This evidence suggests that PD cells are less capable of activating the proper systems to maintain homeostasis which, as highlighted earlier, is already altered at basal level. These results might explain why PD fibroblasts show higher mortality rate as compared to controls.

As for mitochondrial function, the data on fibroblast bioenergetic behavior demonstrates that exposure to even lower concentrations of rotenone can discriminate PD cells. In fact, when fibroblast respiratory capacity was challenged with the toxin, we found that the drop (i.e. reduction) of respiration was significantly smaller in PD cells as compared to controls, consistent with the role that defective complex I activity might play in PD fibroblasts' bioenergetics. Indeed, complex I deficiency is one of the milestones in PD pathogenesis and evidence has been collected from human brains and specimens together with several cellular and animal models of PD [45]. Also, in previous studies performed on peripheral cells obtained from patients, reduced mitochondrial complex I activity was identified as a critical feature to discriminate PD samples from controls, as well as patients with other neurodegenerative disorders [8,9,46,47]. Furthermore, no striking evidence has so far demonstrated that complex I activity and more generally mitochondrial respiration is affected by anti-PD therapy, in particular chronic levodopa administration [48]. Previous studies report that complex I activity is altered in platelets derived from untreated PD patients [49,50], while it is
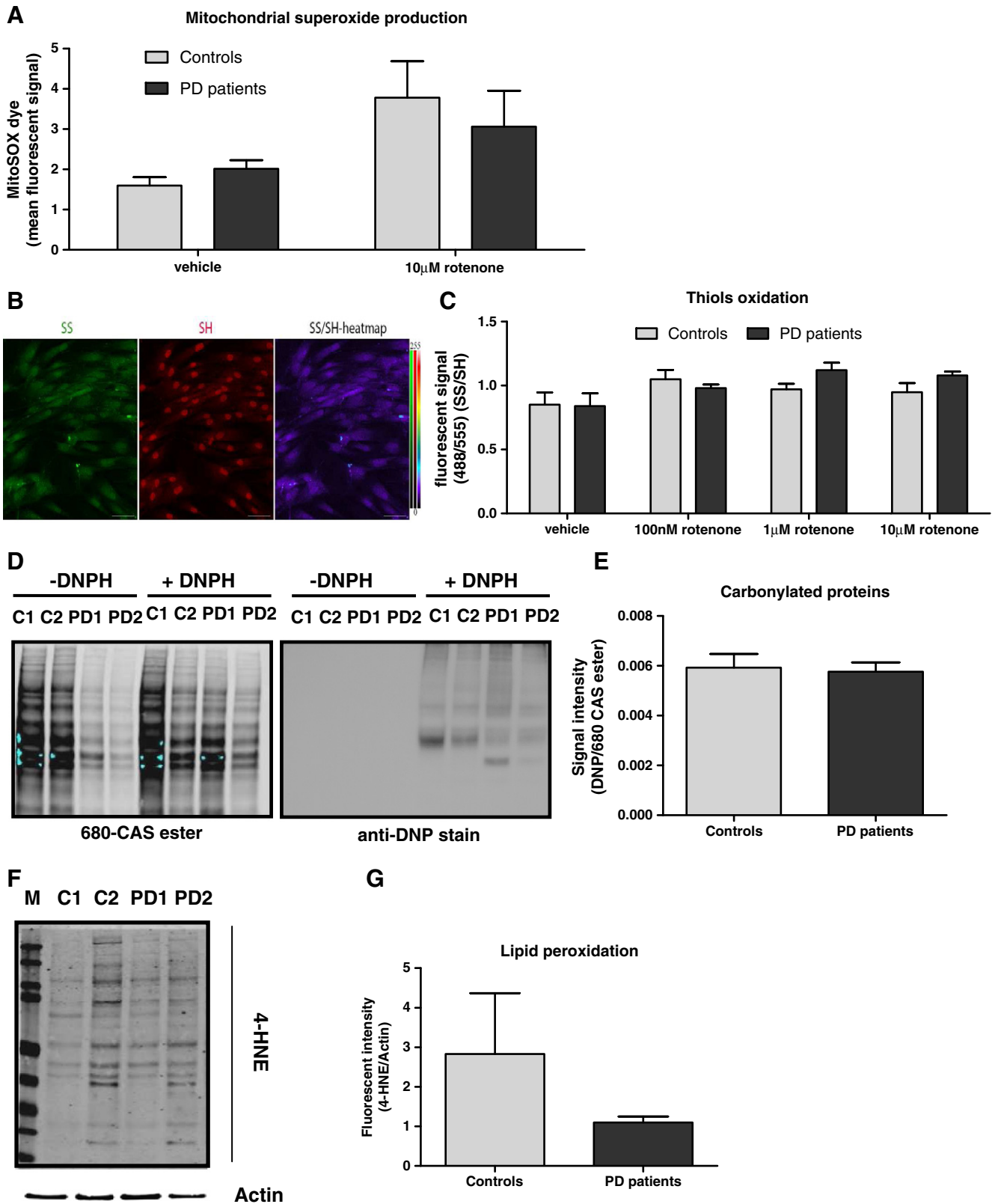
Fig. 5. Markers of intracellular redox state and macromolecule oxidation in control and PD fibroblasts. (A) Levels of mitochondrial superoxide (MitoSOX) production at baseline (vehicle) and after 1 h exposure to 10 μM rotenone, (B) confocal images obtained after the redox immunocytochemistry. Green (AlexaFluor488) and red (AlexaFluor555) signals represent disulfides (SS) and reduced thiols (SH), respectively. The ratio is shown in false colors, as a heat-map. Color scales and scale bars: 20 μm, are reported; (C) redox status of disulfides and thiols (SS/SH) at baseline (vehicle) and after exposure to rotenone (100 nM–1 μM–10 μM); (D) representative OxyBLOT on two control samples (C1–2) and PD samples (PD1–2). Left: membrane stained for Alexa Fluor 680-carboxylic acid succimidyl (CAS) ester, for protein content normalization. Right: membrane with DNPH-free (negative controls) and DNP-positive (carbonyls) lanes; (F) representative membrane blotted with anti-4-Hydroxynonenal (4-HNE) antibody, marker of lipid peroxidation; (E) levels of DNP-antibody and (G) 4-HNE antibody in PD and control fibroblasts (controls: n = 7, PD patients: n = 11). Results are expressed as mean ± sem.

normal and does not correlate with levodopa usage in patients with multiple system atrophy [51]. This evidence further stresses that complex I impairment is a PD-specific pathogenic feature and not a consequence of neurodegeneration and/or pharmacological treatment.

Together with the exacerbated sensitivity to rotenone, we found a blunted response to oxidative phosphorylation uncoupler FCCP in PD fibroblasts, compared to control fibroblasts. In fact, the boost in mitochondrial respiration triggered by FCCP (maximal respiratory capacity) was significantly reduced in PD cells, suggesting that conditions of high energy requirement causing hyperactivation and overload of the electron transport chain are more challenging for PD cells, as already shown by other groups [52,53,42].

5. Conclusions

Our study shows that proteolytic defects and bioenergetic deficits that typically sustain the neurodegenerative process of PD can be detected in fibroblasts from idiopathic PD patients. Also, increased susceptibility to a PD-inducing toxin such as rotenone was detected in PD fibroblasts, thereby supporting the idea that patient-derived cells have a different and less prompt capacity of compensating to stressing conditions, likely because of basal defects such as those affecting UPS activity. These intrinsic deficits might trigger the activation of inefficient responses and enhance cell vulnerability, eventually leading to increased cell mortality rates [54]. Fibroblasts therefore represent a powerful and minimally invasive tool to investigate PD pathogenic mechanisms and, together with other cellular models, might be critical not only for understanding the basis of the selective vulnerability of dopaminergic neurons in PD, but also for discriminating salient features that contribute to the specific susceptibility across different neurodegenerative disorders.

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