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Effect of resveratrol on mitochondrial function: Implications in parkin-associated familiar Parkinson's disease

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ABSTRACT

Mitochondrial dysfunction and oxidative stress occur in Parkinson's disease (PD), but the molecular mechanisms controlling these events are not completely understood. Peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α) is a transcriptional coactivator known as master regulator of mitochondrial functions and oxidative metabolism. Recent studies, including one from our group, have highlighted altered PGC-1α activity and transcriptional deregulation of its target genes in PD pathogenesis suggesting it as a new potential therapeutic target. Resveratrol, a natural polyphenolic compound proved to improve mitochondrial activity through the activation of several metabolic sensors resulting in PGC-1α activation. Here we have tested in vitro the effect of resveratrol treatment on primary fibroblast cultures from two patients with early-onset PD linked to different *Park2* mutations. We show that resveratrol regulates energy homeostasis through activation of AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1) and raise of mRNA expression of a number of PGC-1α's target genes resulting in enhanced mitochondrial oxidative function, likely related to a decrease of oxidative stress and to an increase of mitochondrial biogenesis. The functional impact of resveratrol treatment encompassed an increase of complex I and citrate synthase activities, basal oxygen consumption, and mitochondrial ATP production and a decrease in lactate content, thus supporting a switch from glycolytic to oxidative metabolism. Moreover, resveratrol treatment caused an enhanced macro-autophagic flux through activation of an LC3-independent pathway. Our results, obtained in early-onset PD fibroblasts, suggest that resveratrol may have potential clinical application in selected cases of PD-affected patients.

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1. Introduction

The second most common neurodegenerative disease following Alzheimer's disease, is Parkinson's disease (PD), a progressive disorder, characterized by the loss of dopaminergic neurons in the substantia nigra. The current therapy is symptomatic and does not affect the course of the disease. Although the pathogenesis of PD is likely to be multifactorial and the majority of cases are sporadic, the study of genes linked

Abbreviations: 6-OHDA, 6-hydroxydopamine; AMPK, AMP activated protein kinase; cAMP, cyclic adenosine monophosphate; CAT, catalase; CI, complex I; CII, complex II; CIV, complex IV; COX, cytochrome c oxidase; DMSO, dimethyl sulfoxide; DNP, dinitrophenol; DCF, dichlorodihydrofluorescein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H3, histone3; LC3, protein 1 light chain 3; MPTP, 1-methyl-4,1,2,3,6-tetrahydropyridine; NAD, nicotinamide adenine dinucleotide; OCR, oxygen consumption rates; OXPHOS, oxidative phosphorylation system; PD, Parkinson's disease; PGC-1α, peroxisome proliferator-activated receptor gamma-coactivator 1-alpha; PINK1, PTEN-induced putative kinase 1; RC, respiratory chain; ROS, Reactive oxygen species; SIRT1, NAD-dependent deacetylase sirtuin-1; SOD2, mitochondrial superoxide dismutase; TFAM, mitochondrial transcription factor A

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to rare hereditary forms of PD demonstrates, in patients' fibroblasts, abnormalities in convergent pathways involving oxidative stress, mitochondrial dysfunction and protein aggregation [1–5]. *PARK2* gene mutations are responsible, in humans, for an autosomal recessive form of early-onset parkinsonism. *PARK2* encodes for parkin, a protein of no precisely defined function, which, however, is a component of a multiprotein E3 ubiquitin ligase complex that in turn is part of the ubiquitin–proteasome system targeting protein for degradation. The loss of the normal function of parkin leads to impaired clearance of damaged mitochondria [6]. Recently, Shin and colleagues demonstrated that the progressive loss of dopaminergic neurons in knockout mice models of parkin deficiency resulted in increased level of PARIS, a new parkin interacting substrate. PARIS, which is up regulated in the brain of patients, turned out to be a corepressor of peroxisome-proliferator-activated receptor gamma coactivator PGC-1α (PGC-1α) expression [7]. PGC-1α [8,9] is member of a family of transcription coactivators playing a central role in the regulation of mitochondrial biogenesis and cellular energy metabolism [10,11]. A genome-wide expression meta-analysis study showed abnormal expression of known targets of PGC-1α in PD patients manifesting in the early stages of PD [12]. Accordingly we have previously shown in *PARK2*-mutant fibroblasts altered PGC-1α expression leading to transcriptional deregulation of target genes [1].

Recently, Mudò et al. have shown that transgenic mice overexpressing PGC-1α in dopaminergic neurons are resistant against cell degeneration induced by the neurotoxin 1-methyl-4,1,2,3,6-tetrahydropyridine (MPTP) [13]. Direct evidence for the therapeutic potential of PGC-1α has come from studies in cell culture and animal models [14,15].

PGC-1α expression can be activated by specific compounds able to modulate its upstream regulators, such as NAD-dependent deacetylase sirtuin-1 (SIRT1) and AMP-activated protein kinase (AMPK). Resveratrol, a natural polyphenolic compound found in a wide variety of plant species, induces expression of genes involved in mitochondrial biogenesis, oxidative phosphorylation and endogenous antioxidant defense by modulation of cell signaling pathways that control cell homeostasis [16–21]. Although the effects of resveratrol in PD are uncertain, it seems to protect against different cytotoxic neurotoxins such as MPTP [13,22,23] and 6-hydroxydopamine (6-OHDA) [24–26]. Furthermore, resveratrol protects SH-SY5Y against dopamine-induced cytotoxicity [27] and neuronal cells against toxicity arising from the aggregation-prone protein, alpha-synuclein [28].

In keeping with these premises, we supposed that resveratrol could alleviate mitochondrial dysfunctions induced by impairment of parkin function and tested this hypothesis using fibroblast cultures from two patients affected by an early-onset form of PD with *PARK2* heterozygous mutations. Treatment of PD patient-derived cells with resveratrol induced a partial rescue of mitochondrial functions likely linked to the activation of the AMPK/SIRT1/PGC-1α pathway suggesting a potential beneficial action of resveratrol treatment in PD.

2. Materials and methods

2.1. Patients

The diagnosis of PD was made according to the UK Brain Bank criteria: all patients underwent neurological examination including the motor part of the Unified Parkinson's Disease Rating Scale (UPDRS III) and Hoen–Yahr Scale (H&Y). The parkin1 patient was previously described in Pacelli et al., labeled as P2 [1]. The parkin2 patient was a 48 year old woman with a positive familiar history of PD (one sister and one brother) and an age at onset of 31 years; symptoms of onset were bradykinesia and rigidity of right arm followed by lower limb involvement, one year later, and then controlateral diffusion. Rest tremor was only rarely reported. Treatment at time of examination included levodopa 700 mg and pramipexole 2.1 mg with an excellent response but presence of severe ON dyskinesias. UPDRS III in OFF state was 48 with an H&Y of 3. No atypical signs were found at neurological examination. Genetic analysis of parkin2, indicated as IT-021-007, was reported in [29].

2.2. Skin fibroblasts and culture conditions

Primary fibroblasts from two PD patients (parkin1 and parkin2) and from parental healthy control (parkin1's mother, control1), were obtained by explants from skin punch biopsy, after informed consent. Adult normal human dermal fibroblasts (control2), purchased from Lonza Walkersville Inc., have been utilized as unrelated control. Cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine, 1% (v/v) penicillin/streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2. All experiments were performed on cells with similar passage numbers, ranging from 5 to 14, to avoid an artifact due to senescence, known to occur at passage numbers greater than 30. In the passage range used, fibroblasts were β-Gal negative. For treatment conditions, the media were removed and the cells were incubated subsequently with fresh media containing 25 μM resveratrol (Sigma, R5010) or with equivalent volume of dimethyl sulfoxide (0.02% DMSO, vehicle). In time–response treatments, a parallel experiment exposing the cells to DMSO was set as a control to calibrate the observed results (data not shown). To determine cell viability in our treatment conditions, the colorimetric MTT assay was used according to the manufacture's instruction.

2.3. Measurement of endogenous respiration rates in intact cells

Mitochondrial oxygen consumption was measured polarographically with a Clark-type oxygen electrode in a water-jacketed chamber (Hansatech Instruments, Norfolk, UK), magnetically stirred at 37 °C as previously described [30]. Briefly, exponentially growing cells, fluid changed the day before the measurement, were collected by trypsinization and centrifugation, washed once in TD (0.137 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris–HCl, pH 7.4), resuspended in the same buffer previously air equilibrated at 37 °C, and transferred into a polarographic chamber, at a final concentration of 1 to $3x10^6$ cells per ml. After the native endogenous O_2 consumption rate was recorded, dinitrophenol (DNP) was added at a concentration of 30 μM, followed by 20 nM antimycin A to inhibit the upstream segment of the RC.

2.4. Measurement of total cellular ATP

The fibroblast cells were grown in six-well plates. Once the cells were at 75% confluence, ATP level was measured in untreated and resveratrol-treated cells incubated for 48 h with 25 μM resveratrol. Where indicated, 5 μM oligomycin was added to the cells during the last hour of resveratrol treatment. After incubation the cells were collected by trypsinization and centrifugation at 500 ×*g* and then resuspended in phosphate-buffered saline, pH 7.4. Cellular ATP content was determined using the PerkinElmer "ATPlite" kit (PerkinElmer) according to the manufacturer's instructions measurements were performed on a Victor 2030 Explorer (PerkinElmer) and normalized on protein content.

2.5. OXPHOS enzyme and citrate synthase activities measurements

Cells, collected by trypsinization and centrifugation, were resuspended in hypotonic medium (25 mM potassium phosphate, pH 7.2, 5 mM $MgCl_2$), supplemented with anti-proteases cocktail tablet (Roche, Basel, CH). In order to allow complete accessibility of substrates to the inner mitochondrial membrane enzymes, samples were freeze-thawed three times, gently shaken and then resuspended in the assay buffer. CI (NADH-ubiquinone oxidoreductase, rotenone sensitive), CII (Succinate-CoQ oxidoreductase, malonate sensitive), CIV (cytochrome

c oxidase, KCN sensitive) and citrate synthase activities were measured spectrophotometrically with a Beckman DU7400 equipped with a rapid-mixing apparatus at 30 °C essentially as previously described [1].

2.6. NAD^+ and NADH cellular level measurement

Cells, collected by trypsinization and centrifugation, after protein determination, were suspended in 15% $HClO_4$ (NAD^+ extraction) or 0.2 M NaOH (NADH extraction). After 10 min, the suspensions were neutralized by adding 0.1 M KOH (NAD^+ extraction) or 0.1 M HCl (NADH extraction). After centrifugation, the supernatants were collected and used immediately. NAD or NADH concentrations were measured in cyclic enzyme reaction system in which alcohol dehydrogenase reduces dichlorophenol indolphenol (DCIPIP) through the intermediation of phenazine methosulfate (PMS). The reaction mixture consisted of 0.63 ml of 100 mM phosphate Buffer (pH 7.5), 0.03 ml of 30 mM phenazine methosulphate (PMS), 0.04 ml of 0.6 mM dichlorophenol indolphenol (DCIPIP), 0.1 ml of 95% ethanol, 5 units ADH. The reaction was started by the addition of 200–300 μg of the sample. Reduction of the blue-colored DCPIP to colorless $DCPIPH_2$ was measured by recording the decrease in absorbance at 600 nm. The concentration of NADH and NAD^+ in each extract was determined by comparing sample values to standard curves generated from samples containing known amounts of NADH and NAD^+ that had been cycled under identical conditions as the samples.

2.7. Real-time PCR

Purification of total RNA from fibroblasts was carried out using RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. One microgram of total RNA was then reverse-transcribed to generate cDNA for PCR by using iScript cDNA Synthesis kit (Bio-Rad). q-PCR on cDNA was performed as previously described [1], using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin as internal control. mtDNA content was assayed by q-PCR using 100 ng of total DNA, isolated with DNA extraction kit (EuroGOLD Tissue DNA mini Kit), with primers amplifying the cytochrome b region and normalized to the 18 S nuclear DNA. Relative quantification was performed by using the ΔΔCT method. Validated primers for q-PCR are reported in Supplemental Materials (Table I).

2.8. Western blot analysis

Cells in hypotonic medium supplemented with antiproteases cocktail tablet (Roche, Basel, CH) were freeze-thawed three times. Total cell proteins (30 μg) were separated on a 12% Tris-Tricine SDS–PAGE and transferred onto nitrocellulose membrane. Western blot analysis was performed using the specified primary antibodies against AMPK and p-AMPKα (Thr172) (Cell Signaling Technology), and acetyl-lysine (clone 4G12, Merck Millipore) according to the manufacturer's instructions. For PGC-1α protein detection, total cell proteins (45 μg) were separated on an 8% Tris-Tricine SDS–PAGE and transferred onto nitrocellulose membrane. Polyclonal PGC-1α primary antibody (Santa Cruz Biotechnology) was used according to the manufacturer's suggested concentrations. For LC3 detection, total cell proteins (45 μg) were separated on a 12% Tris-Glycine SDS–PAGE and transferred onto nitrocellulose membrane. Western blot analysis was performed by using a specific antibody against LC3B (Cell Signaling Technology).

2.9. Microscopy analysis

Laser scanning confocal microscopy (LSCM) live cell imaging of ROS was determined in cells cultured at low density on fibronectin-coated 35-mm glass-bottom dishes incubated for 20–30 min at 37 °C with 10 μM 2,7-dichlorofluorescin diacetate, which is converted to dichlorofluorescein by intracellular esterases, or with 5 μM MitoSox (from Molecular Probes, Eugene, OR). Stained cells were washed with PBS and examined with a Nikon TE 2000 microscope (images collected using a 60× objective [1.4 NA]) coupled to a Radiance 2100 dual-laser LSCM system (Bio-Rad); dichlorofluorescein green fluorescence was elicited with the Ar–Kr laser beam (λ_{ex} 488 nm), MitoSox red fluorescence was elicited with the He–Ne laser beam (λ_{ex} 543 nm). Acquisition, storage, and analysis of data were performed with LaserSharp and LaserPix software from Bio-Rad or ImageJ version 1.37. For fluorescence microscopy analysis, fibroblast cells, were washed two times in PBS and incubated with the Cyto-ID® Green Detection Reagent and Hoechst 33342 Nuclear Stain (Cyto-ID Autophagy Detection Kit, Enzo Life Science, NY, USA) according to the manufacturer's instructions, mounted in Vectashield (Vector) and examined with an Olympus photomicroscope (Olympus Italia, Rozzano, Italy) using 63× objective lenses with either 1× zoom factors. Images were analyzed, digitally recorded and stored as TIFF files using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA, USA). Morphometric analysis of labeled areas were evaluated on twenty randomly selected fields for each experimental group, observed at ×630 magnification with an Olympus photomicroscope, using Image Analysis software (Olympus Italia, Rozzano, Italy).

2.10. Quantitative determination of intracellular ROS level

Quantitative analysis of ROS level was determined using the cell permeant probe 2′-7′dichlorodihydrofluorescin diacetate (H_2DCFDA). The ROS-dependent oxidation of the fluorescent probe (507 nm excitation and 530 nm emission wavelength) was measured by a Jasco FP6200 spectrofluorometer as described in [1].

2.11. siRNA mediated parkin knockdown

Control1 fibroblasts at 60–70% of confluence were transiently transfected for 48 h with 20 nM of small interfering RNAs (siRNAs) specific for human *parkin* gene (Parkin siRNA), siRNA against human GAPDH as a positive control and a mixture of 4 scrambled siRNAs (scramble siRNA) as a negative control or with the transfection reagent alone (Control siRNA) according to the manufacturers' instructions (SMART-pool; Dharmacon RNA Technologies, Lafayette, CO).

2.12. Statistical analysis

Data of quantitative measurements are expressed as means ± SEM, except for fluorescence microscopy analysis (±SD), of more than three independent experiments. Statistical analyses were performed using the unpaired Student's *t* test or, where specified, the one-way or two-way ANOVA followed by Bonferroni post-hoc test; a p value < 0.05 was set for statistically significant differences.

3. Results

In this study we used cultured skin fibroblasts from two unrelated patients affected by an early-onset PD, labeled as parkin1, characterized in a previous study [1] and parkin2, with the *PARK2* heterozygous mutations del-exon2-3/del-exon3 and del-exon7-9/Glu409X, respectively. Parental healthy control, displaying the heterozygous del-exon2-3, (parkin1's mother, control1) and unrelated control consisting in normal adult human dermal fibroblasts (control2), have been also included in this study. The Western blot analysis of parkin protein expression revealed the complete absence of the 50 kDa full-length protein in the fibroblasts of both patients and a comparable amount in control1 and in control2 fibroblasts (Fig. S1). Moreover to mimic the pathogenic phenotype of parkin deficiency we characterized knockdown parkin fibroblasts (Fig. S2).

3.1. Resveratrol enhances mitochondrial oxidative capacity

To test the effect of resveratrol treatment on the mitochondrial oxidative capacity, we measured oxygen consumption rates (OCR) by endogenous substrates in intact cells cultured in the presence of 25 μM resveratrol or vehicle for 48 h (Fig. 1A). Resveratrol concentration and time exposure were chosen according to preliminary tryouts showing no cytotoxicity at 24 and 48 h of treatment with 25 μM of resveratrol (Fig. S3). As shown in Fig. 1 the basal and the DNP-uncoupled OCR was significantly lower in patients' fibroblasts as compared to the control values (see also Fig. S4; one-way ANOVA test: F_{Basal} (3, 20) = 31.07; $P < 0.0001$; $F_{DNP\text{-}uncoupled}$ (3, 20) = 39.29; $P < 0.0001$) with parkin1 showing a more compromised respiratory phenotype as compared with parkin2, which exhibited a relatively milder impairment of respiration. A little but significant decrease of the OCR has been detected also in Parkin-siRNA fibroblasts (Fig. 1B). Treatment of fibroblasts with resveratrol led to an appreciable significant increase of basal OCR in control1, parkin1 and parkin2 cells as compared to the fibroblasts incubated with vehicle. The maximal OCR, achieved in the presence of the mitochondrial protonophore DNP, was also increased in parkin1 and parkin2 cells.

The maximal OCR is an index of the content of the respiratory chain (RC) complexes or of the complex controlling the overall rate of respiration. Thus the effect of resveratrol would suggest an up-regulation of the RC complexes' biogenesis. It is important to note that resveratrol treatment was able to rescue substantially the mitochondrial respiratory function in parkin2 whereas the parkin1 oxygen consumption capacity remained below the OCR of the untreated controls.

We have previously reported that parkin1's fibroblasts displayed an up-regulated glycolytic ATP production as a consequence of a defective oxidative phosphorylation (OXPHOS) [1]. To investigate the relative contribution of mitochondrial OXPHOS and glycolysis to ATP production, we determined the intracellular basal ATP content in untreated and resveratrol-treated fibroblasts, both in the absence and in the presence of oligomycin, a specific inhibitor of the mitochondrial F1F0-ATP-synthase. Resveratrol treatment for 48 h induced a significant increase of the oligomycin-sensitive ATP content (Fig. 2A) in controls' and patients' cells as compared to the vehicle-treated fibroblasts. In

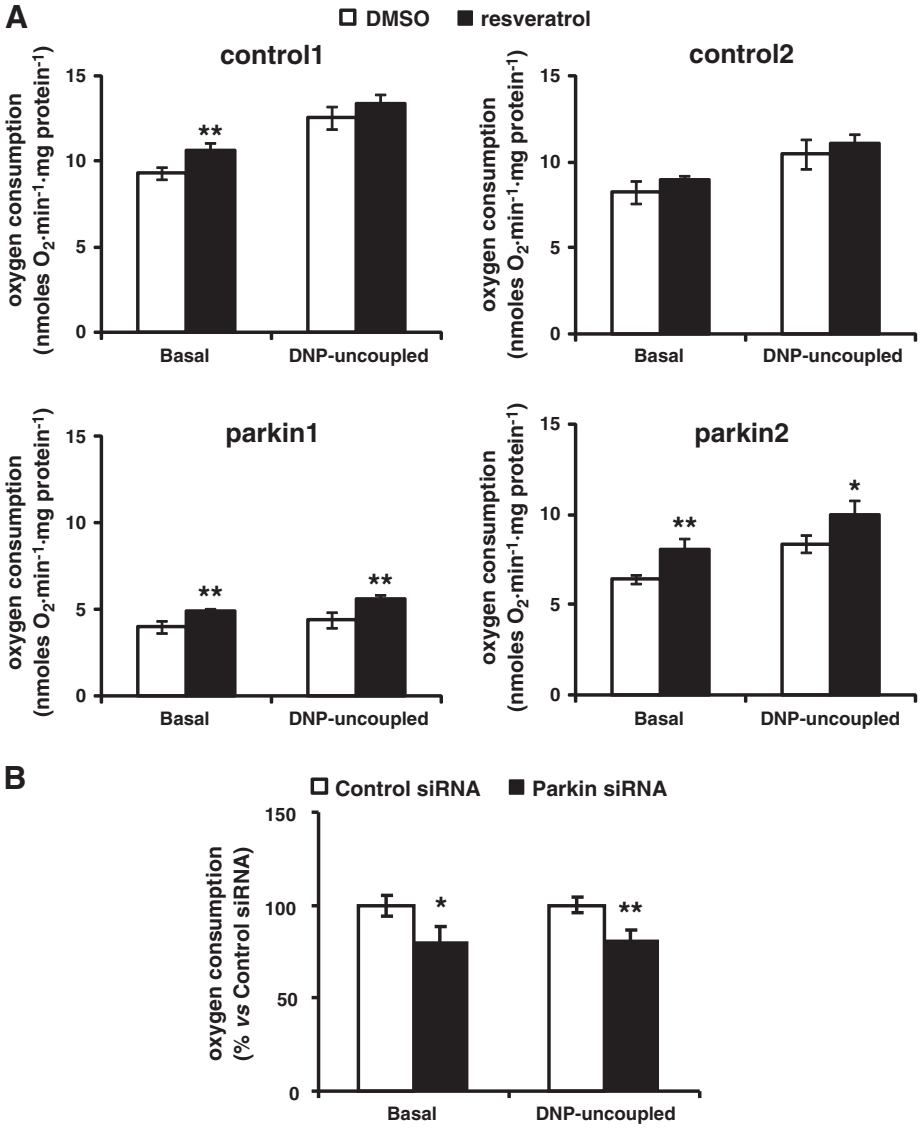

Fig. 1. Respiration rates in intact cells. (A) Effect of resveratrol on respiration rates in the presence of endogenous substrates (Basal) and DNP (30 μM)-uncoupler agent in intact cells from control1, control2, parkin1 and parkin2 fibroblasts, exposed to either vehicle (DMSO) or 25 μM resveratrol for 48 h. The values reported represent the means ± SEM and significance was calculated with Student's *t* test; n ≥ 5 under each condition; *p < 0.05, **p < 0.005 vs vehicle-treated cells. (B) Respiration rates in the presence of endogenous substrates (Basal) and DNP (30 μM)-uncoupler agent in Control siRNA and Parkin-siRNA cells. Data, means ± SEM are expressed as percentage of Control siRNA; n = 3 under each condition. Significance was calculated with Student's *t* test; *p < 0.05, **p < 0.005 vs Control siRNA cells. For more details see Materials and methods.

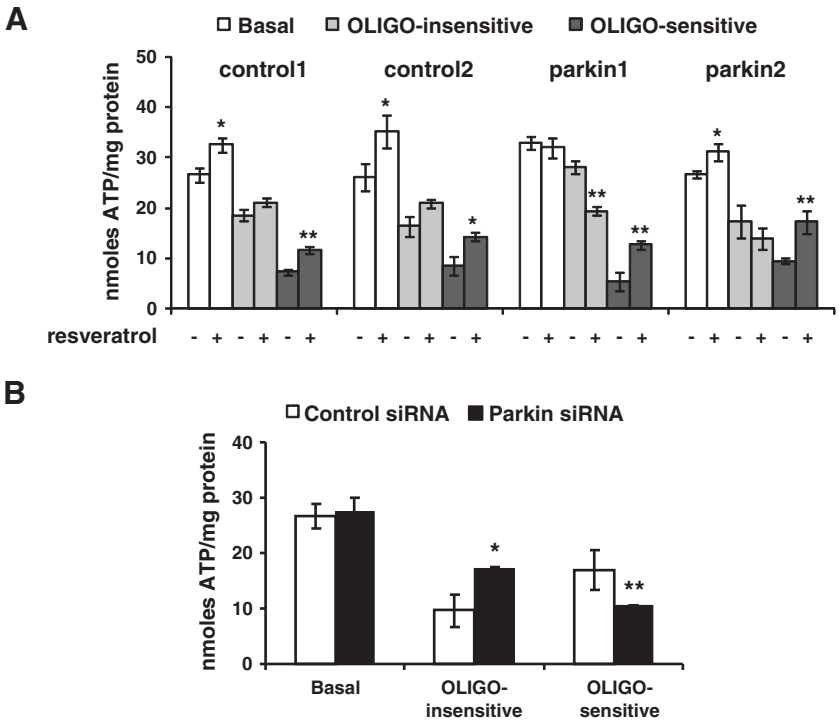

Fig. 2. Cellular ATP content. (A) Effect of resveratrol on the total cellular ATP content measured under basal conditions (Basal) in controls' and patients' fibroblasts exposed to either vehicle or 25 μM resveratrol for 48 h. The oligomycin-resistant (OLIGO-insensitive) ATP contents were determined in cells incubated with oligomycin 5 μM during the last hour of resveratrol treatment. The oligomycin sensitive (OLIGO-sensitive) ATP production was calculated by subtracting the oligomycin-resistant value from the basal ATP content. The values represent the means ± SEM and significance was calculated with Student's *t* test; n = 5 under each condition; *p < 0.05, **p < 0.005 vs vehicle-treated cells. (B) ATP basal cellular content, oligomycin-insensitive and oligomycin-sensitive ATP level in Control siRNA and Parkin-siRNA cells. The values represent the means ± SEM and significance was calculated with Student's *t* test; n = 3 under each condition; *p < 0.05, **p < 0.005 vs Control siRNA. For more details see Materials and methods.

parkin1 patient, the basal content of ATP did not change after resveratrol treatment, but a significant decrease in the ATP level was observed in the presence of oligomycin. Although not reaching a statistical significance a similar trend was also observed in parkin2. This supports the hypothesis that, in patients' fibroblasts, a resveratrol dependent switch from glycolytic to oxidative metabolism occurred. Consistently, a significant decrease of extracellular lactate measured under basal respiratory conditions in resveratrol-treated control2, parkin1 and parkin2, as compared to the vehicle-treated fibroblasts was observed (Fig. S5).

When the basal cellular content of ATP was measured in Parkin-siRNA fibroblasts a larger reliance on glycolytic ATP resulted when compared with control siRNA cells (Fig. 2B) matching what observed in parkin1 with respect to control1 and control2 samples.

Fibroblasts from subjects carrying mutated PD-related genes were repeatedly reported to exhibit an altered redox balance. Accordingly, Fig. 3A shows, by confocal microscopy imaging, that parkin1 and parkin2 fibroblasts displayed a significantly much higher level of the DCF-fluorescence, a commonly used probe to assess the intracellular redox state, when compared with control cells. Likewise, parkin siRNA cells displayed a higher level of the DCF-related fluorescence when compared with Control siRNA cells (Fig. 3B).

Noticeably, resveratrol treatment abrogated almost completely the ROS-dependent DCF fluorescent signal in both patients' fibroblasts.

Since mitochondria are a major source of ROS, MitoSox an organelle-selective probe was used to reassess the intracellular ROS-generating compartment. As illustrated in Fig. 4, parkin1 and parkin2 fibroblasts showed a significant higher probe-related punctuate fluorescence as compared with control cells whereby pointing to alteration of the mitochondrial oxidative metabolism as culprit of the observed redox unbalance in PD-derived fibroblast. Intriguingly, while the ROS-mediated fluorescence was completely abrogated in all the cellular samples by treatment with the synthetic antioxidant ebselen, treatment with resveratrol was effective in reducing ROS production in patients' fibroblasts leaving unchanged the basal level of ROS in control cells. This observation suggests an antioxidant activity of resveratrol not simply explainable in terms of ROS-scavenging specifically accomplished in the cell phenotype of the PD-derived fibroblasts.

Mitochondrial ROS production in PD is frequently associated with defective activity of the respiratory chain complexes with complex I (CI) recognized as the major "ROS-genic" site. In our previous study impaired activity of RC complexes in parkin-mutant fibroblasts has been already reported [1]. Consistently, Parkin-siRNA treated fibroblasts displayed a highly significant decrease of CI and of citrate synthase (a key component of the TCA cycle) activity compared to Control siRNA, while the CIV (complex IV) activity was not significantly changed (Fig. S6).

To gain deeper insights into the resveratrol effect on the respiratory system, the specific enzymatic activity of mitochondrial Complex I (CI), Complex II (CII) and Complex IV (CIV) and of citrate synthase were measured. As shown in Fig. 5 the comparative analysis of the RC complexes' activities confirmed in both parkin1 and parkin2 a significant depression of CI as compared with control1 and control2. Most notably, resveratrol treatment increased significantly and specifically the CI activity, in both controls' and patients' cells as compared to the fibroblasts cultures incubated with vehicle. Importantly, in both patients' fibroblasts resveratrol treatment raised the CI activity to the basal levels of the untreated control fibroblasts and interestingly, citrate synthase activity, which is considered also an index of the mitochondrial mass, was specifically increased.

To evaluate if resveratrol treatment resulted in an increased mitochondrial biogenesis, we measured mitochondrial DNA (mtDNA) content. As shown in Fig. 6, resveratrol treatment induced a significant increase, although of small entity, of the relative mtDNA content (mtDNA/nDNA) measured by q-PCR of the mtDNA-harbored cytochrome *b* gene both in control and patients' fibroblasts; assessment of the relative mtDNA content by detection of a different mtDNA-

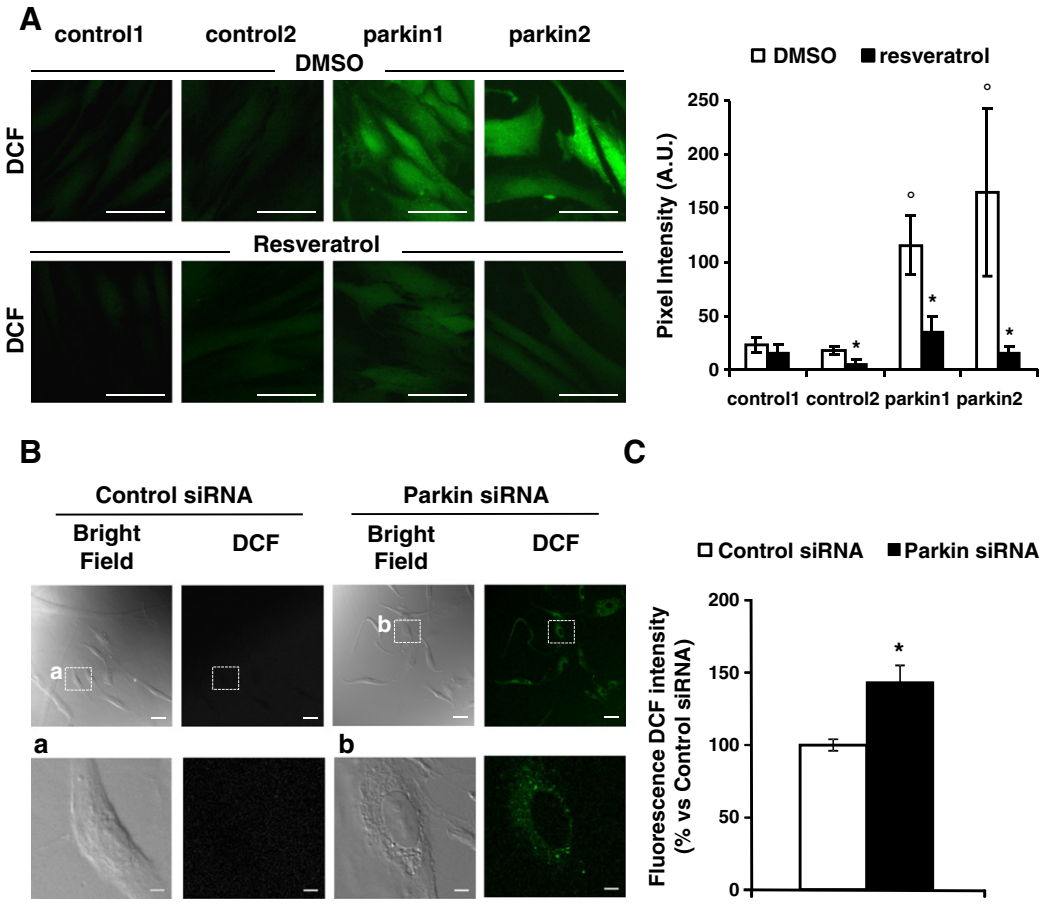

Fig. 3. Intracellular ROS content. Cultured controls' and patients' fibroblasts were incubated for 48 h with vehicle (DMSO) or 25 μM resveratrol then treated with DCFH-DA for 30 min and analyzed by confocal microscopy as described in Materials and methods. (A) Representative images of three experiments are shown Scale bar, 10 μm. The graph displays the statistical analysis of the DCF-related mean pixel intensity with the bars indicating the values averaged from 8 to 10 different optical field under each condition (±SEM). Significance was calculated with Student's *t* test; n = 3 under each condition; *p < 0.005 vs vehicle-treated cells, °p < 0.001 for parkin1 and parkin2 vs vehicle-treated control1. Qualitative (B) and quantitative (C) analysis of ROS cellular content in Parkin siRNA and Control siRNA cells. (B) Confocal microscopy representative images under bright field and fluorescence mode. Scale Bar: 40 μm. Enlarged details from the optical field indicated as "a" and "b", are also shown. Scale bar: 5 μm. (C) Spectrofluorimetric measurements of intracellular ROS content in cells loaded with DCFH-DA. Data, means ± SEM are expressed as percentage of Control siRNA; n = 3 under each condition. Significance was calculated with Student's *t* test; *p < 0.05 vs Control siRNA. For more details see Materials and methods.

harbored gene (i.e. ND1) resulted in comparable result (data not shown). In spite of the observed significant resveratrol-mediated increase of the mtDNA/nDNA ratio, resveratrol-treatment induced a significant enhancement of mtDNA-encoded protein synthesis only in parkin1 fibroblasts whose basal translation was, as previously reported, consistently lower with respect to the control1 fibroblasts (Fig. S7).

3.2. Resveratrol modulates AMPK and the NAD^+/NADH ratio

Growing evidences point to the fuel sensor AMP-activated protein kinase (AMPK) as a key mediator of the metabolic effects of resveratrol [31]. Therefore, we monitored the Thr^{172}-phosphorylation-mediated activation of AMPK in resveratrol-treated fibroblasts using a specific antibody. Fig. 7 shows that the basal p-AMPK/AMPK ratio was significantly lower in both patients' fibroblasts as compared with control cells. Eight hours of resveratrol treatment induced a significant increase of the p-AMPK/AMPK ratio in controls' and patients' cells as compared to the fibroblasts cultures incubated with vehicle, with no changes in the total AMPK protein level. After 24 h of resveratrol treatment the level of AMPK-phosphorylation remained higher than the basal values.

Since it has been reported that resveratrol enhances the NAD^+/NADH ratio, in an AMPK-dependent manner [31–34], we measured, at 8 and 24 h of resveratrol treatment, the steady-state cells content of NAD^+ and NADH. As compared with the control fibroblasts the basal level of NAD^+ and the NAD^+/NADH ratio were significantly lower in both patients' fibroblast (Fig. 8A; one-way ANOVA test: F_{NAD+} (3, 31) = 13.85; P < 0.0001; $F_{NAD+/NADH}$ (3, 16) = 8.284; P = 0.0021). The total cellular NAD content was also significantly reduced (by about 50%) with respect to control fibroblasts (data not shown). Following resveratrol treatment (Fig. 8B), a significant increase of the NAD^+/NADH ratio in controls' and patients' cells as compared to the fibroblasts cultures incubated with vehicle, was observed after 8 h incubation, matching the phosphorylation state of AMPK. At the same time-point only in the control cells a transient increase of the total amount of NAD following resveratrol treatment was observed suggesting induction of NAD biosynthesis (not shown) [35]. The NAD^+/NADH ratio returned to basal levels after 24 h incubation in both patients, but not in control cells where it remained higher than the basal values (Fig. 8B; two-way ANOVA test: F_{NAD+} (2, 45) = 19.93; P < 0.0001; $F_{NAD+/NADH}$ (2, 27) = 40.50; P < 0.0001). These data suggest that interplay between AMPK activation and NAD^+/NADH ratio may occur.

3.3. Resveratrol controls expression and transcriptional activity of PGC-1α

The effect of AMPK activation on mitochondrial energetic functions can be attained through the regulation of a number of transcriptional factors and cofactors [36] among which is PGC-1α [34,37]. In order to assess whether resveratrol enhances expression and activity of PGC-1α, we analyzed the transcription of the PGC-1α gene and of some of its target genes in resveratrol-treated fibroblasts. Transcript level

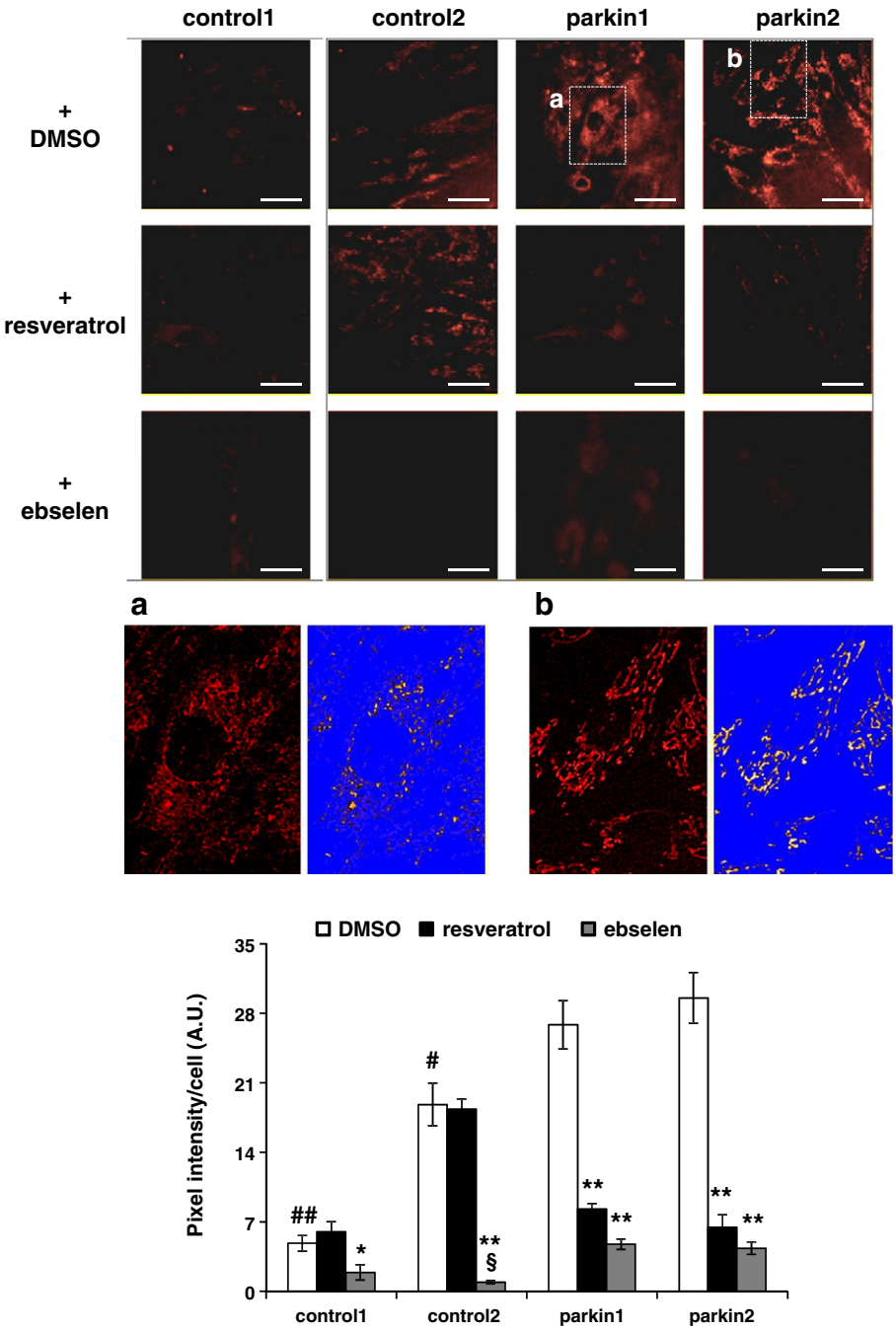

Fig. 4. Effect of resveratrol on mitochondrial ROS content. Confocal microscopy analysis of ROS production. Cultured controls' and patients' fibroblasts were incubated for 48 h with vehicle (DMSO) or 25 μM resveratrol or with 25 μM ebselen then treated with MitoSox for 30 min and analyzed by confocal microscopy as described in Materials and methods. Scale bar, 10 μm. Enlarged details from the optical field of parkin1 and parkin2, indicated as "a" and "b", are also shown with a false-color 3D rendering of the fluorescent signal. Representative images of three experiments are shown. The graph displays the statistical analysis of the MitoSox-related mean pixel intensity/cell with the bars indicating the values averaged from 8 to 10 different optical field under each condition (±SEM) and significance was calculated with Student's *t* test; n = 3 under each condition; #, $p < 0.05$ vs parkin1 and parkin2; ##, $p < 0.001$ vs control2, parkin1, parkin2; *, $p < 0.05$ vs DMSO and resveratrol; §, $P < 0.001$ vs resveratrol; **, $p < 0.001$ vs DMSO.

measurements of PGC-1α revealed abnormal values in parkin1 (10.6-fold higher), already reported [1] and in parkin2 (0.27 fold lower) as compared with control1 fibroblasts (Fig. 9A). Measurement by qRT-PCR revealed a significant decrease in mRNA transcripts of PGC-1α and in some of its down-stream target genes (i.e. TFAM and SOD2) in Parkin siRNA cells compared to Control siRNA (Fig. 10) confirming altered PGC-1a activity and transcriptional deregulation of its target genes in parkin-deficient cells. Resveratrol treatment induced in control1, control2 and parkin2 fibroblasts an increase of mRNA expression of PGC-1α as compared to the fibroblast cultures incubated with vehicle. Conversely, in fibroblasts from patient parkin1, resveratrol treatment induced a significant decrease of PGC-1α mRNA level, which, however, remained at a still higher level (5.3 ± 0.9 fold) as compared to control1 cells. Consistently, Western blot analysis of PGC-1α revealed a higher and lower protein expression in parkin1 and in parkin2, respectively, as compared with control1 fibroblasts. Resveratrol treatment for 24 h induced a slight increase of the protein content, which, however was significant only in parkin2 cells (Fig. 9B). Regardless of the effect on the PGC-1α expression, resveratrol treatment caused transcriptional up-regulation of PGC-1α downstream target

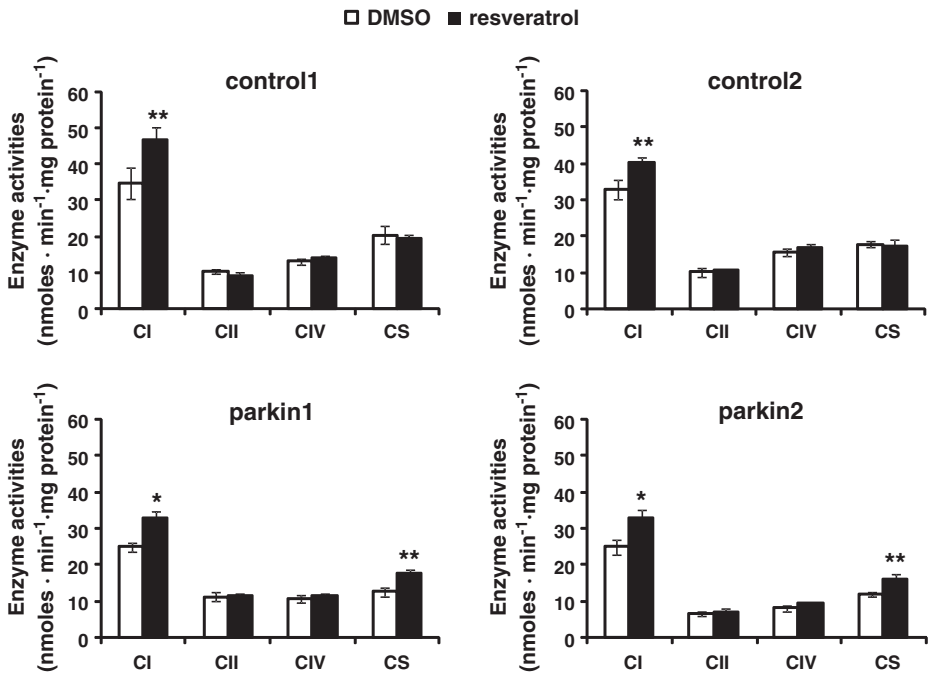

Fig. 5. Effect of resveratrol on mitochondrial respiratory enzyme and citrate synthase activities. Specific enzymatic activities of mitochondrial respiratory chain CI, CII, CIV, and of citrate synthase were measured on total cell lysates from controls' and patients' fibroblasts exposed to either vehicle (DMSO) or 25 μM resveratrol for 48 h. The values represent the means ± SEM and significance was calculated with Student's *t* test; n = 7 under each condition; *p < 0.05, **p < 0.005 vs vehicle-treated cells. For more details see Materials and methods.

genes, directly involved in mitochondrial biogenesis (TFAM, cytochrome *c*, COX I) in both control and patients' cells and coding for the antioxidant enzymes (SOD2, catalase) mainly in control cells (Fig. 9C).

All together, the results presented indicate that the resveratrol-mediated increase of the PGC-1α transcriptional activity, as shown by the up-regulation of its target genes, was not simply related to the PGC-1α expression therefore suggesting a possible effect at a post-translational level.

3.4. Resveratrol controls activity of SIRT1

PGC-1α is modulated through regulation of its expression and of its activity, the latter by posttranslational modifications consisting mainly in AMPK-mediated phosphorylation and SIRT1-mediated deacetylation. The low level of PGC-1α protein in fibroblast cells did not allow us to evaluate directly the acetylation state of the protein. However, the effect of resveratrol on SIRT1 activity was assessed, as reported by several groups [38–40], monitoring the acetylated state of the histone 3 (H3) one of its main downstream target (Fig. 11A). The resveratrol treatment for 24 h induced a significant decrease of acetylated H3 content in controls' and patients' cells compared to the fibroblasts culture incubated

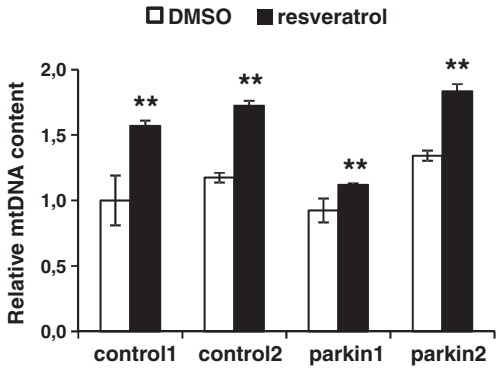

Fig. 6. Effect of resveratrol on mtDNA content. mtDNA from controls' and patients' fibroblasts exposed to either vehicle (DMSO) or 25 μM resveratrol for 24 h was determined by q-PCR amplification of cytochrome *b* normalized to 18S nuclear DNA. Relative expression values were compared with vehicle-treated control1. The values represent the mean ± SEM and significance was calculated with Student's *t* test; n = 4 under each condition; *p < 0.05, **p < 0.005 vs vehicle-treated cells. For more details see Materials and Methods.

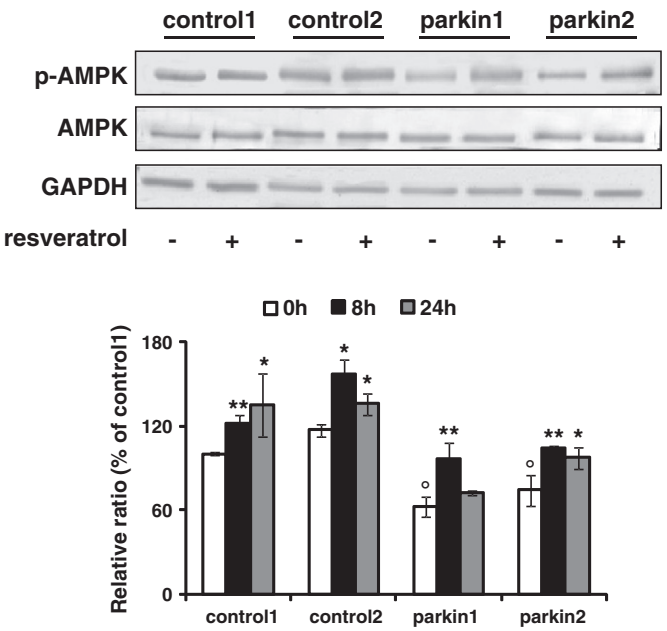

Fig. 7. Effect of resveratrol on p-AMPK/AMPK ratio. Representative Western blot of total AMPK and phosphorylated-AMPK (p-AMPK) levels performed on whole cell lysates from controls' and patients' fibroblasts exposed to either vehicle or 25 μM resveratrol for 8 h. The graph displays the statistical analysis of the p-AMPK/AMPK ratio, from fibroblasts exposed to either vehicle (0 h) or 25 μM resveratrol for 8 and 24 h, calculated by densitometric analysis of band intensity normalized to the corresponding GAPDH level, used as loading control. Data, means ± SEM are expressed as percentage of vehicle-treated control1; n = 4 under each condition. Significance was calculated with Student's *t* test, *p < 0.05, **p < 0.005 vs vehicle-treated cells. °p < 0.05, for parkin1 and parkin2 vs vehicle-treated control1. For more details see Materials and methods.

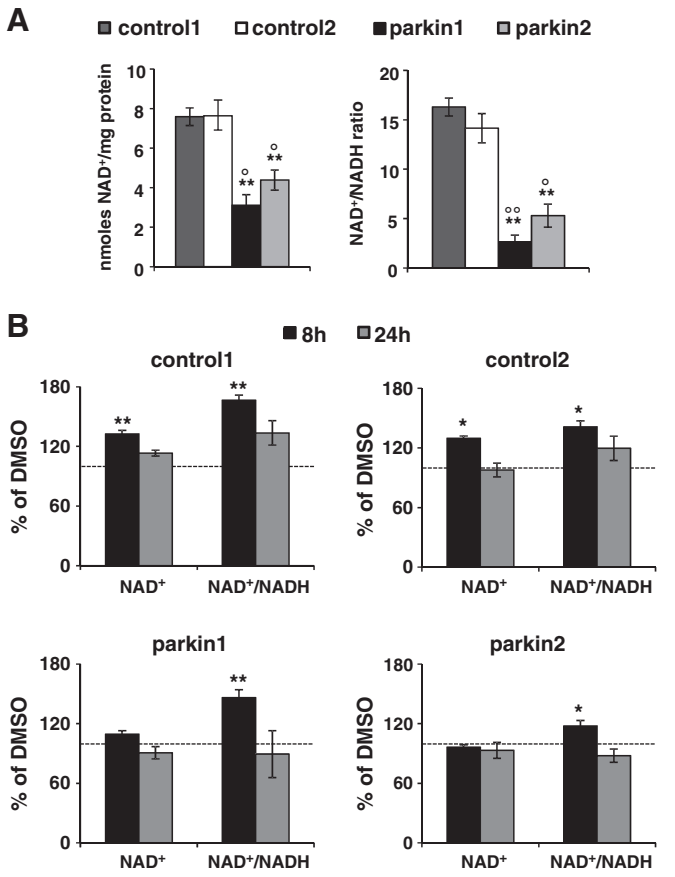

Fig. 8. Effect of resveratrol on NAD^+ level and NAD^+/NADH ratio. (A) Basal NAD^+ level and NAD^+/NADH ratio were determined in controls' and patients' fibroblasts. The values reported represent the means ± SEM and significance was calculated with one-way ANOVA followed by Bonferroni post-hoc test; $n \geq 4$, under each condition; $^{**}p < 0.005$ vs control1; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.005$ vs control2. (B) NAD^+ level and NAD^+/NADH ratio were determined in controls' and patients' fibroblasts incubated for 8 and 24 h with either vehicle or 25 μM resveratrol. Data are expressed as percentage of vehicle-treated cells at the respective time of incubation. The values reported represent the means ± SEM and significance was calculated with two-way ANOVA followed by Bonferroni post-hoc test; $n \geq 3$, under each condition; $^{*}p < 0.05$, $^{**}p < 0.005$ vs vehicle-treated cells. For more details see Materials and methods.

with vehicle, indicating that the SIRT1 deacetylase activity was increased. Most notably, resveratrol treatment caused, also, significant increase of the mRNA levels of SIRT1 (Fig. 11B).

3.5. Resveratrol enhances macro-autophagy

Fine balance of mitochondrial autophagy and biogenesis plays a key role in controlling mitochondrial physiology. Overexpressed parkin enhances mitophagy in FCCP-treated cells through the translocation of tagged parkin to mitochondria and its ubiquitination activity [41]. However, an increase of mitophagic marker has been described as a consequence of parkin knockdown and that parkin-mediated monoubiquitination of Bcl2 enhances the ability of Bcl2 to bind beclin 1 and to suppress autophagy [42]. Thus, depending on subcellular localization and/or target modification, parkin could act to either promote or to downregulate autophagy. Therefore, we sought to verify if resveratrol treatment, in our cell model, was able to modulate autophagy by immunodetecting the microtubule-associated protein 1 light chain 3 (LC3), whose proteolytic and phagosomal phospholipid-ligated products (LC3-I and LC3-II respectively) are widely used as a marker of mammalian autophagy [43]. Fig. 12A shows that the steady-state level of LC3-II did not significantly differ between control and parkin-deficient fibroblasts. When NH_4Cl-treatment was utilized to inhibit acidification of autolysosomes, the LC3-II level increased 2–3 fold in control1, parkin1 and parkin2 cells and to a lower extent in control2. This result would indicate that the autophagic flux in both control and patients' fibroblasts occurs to comparable values somehow lower in control2. To note, 24 h of resveratrol treatment did not affect the LC3-II levels with the exception of parkin2, which in the absence of NH_4Cl displayed a significant increase of the LC3-II content. Intriguingly, when the autophagosome content was visualized using a selective membrane-bound fluorescent probe a different scenario was observed. In contrast with the LC3-II profile the basal number of phagosomes was significantly higher in both patients' fibroblasts as compared with the control cells. Moreover, 24 h of resveratrol treatment induced a large enhancement of the fluorescent signal indicative of a strong induction of autophagy in control1 and control2 but to a lower extent in parkin2 fibroblasts (Fig. 12B) whereas parkin1 cells were resveratrol-insensitive. These results would be consistent with the occurrence of both LC3-dependent and LC3-independent macroautophagic pathways, as recently suggested [44], with the latter being promotable by resveratrol in a parkin context.

Since mitophagy appears to be triggered by a decrease of membrane potential in damaged and fragmented mitochondria [41] we assessed the mitochondrial membrane potential by using TMRE, a fluorescent probe that accumulates in mitochondria in a membrane potential-driven manner. The results of the image analysis are presented in the Supplementary Fig. S8 and show that the averaged mean fluorescence/cell was not significantly different between controls' and patients' samples; also the standard deviations were of the same entity thus indicating a similar distribution of the pixel intensities and therefore ruling out the occurrence of a larger sub-population of mitochondria with low potential in patients' fibroblasts. Resveratrol treatment did not change the membrane potential-related fluorescent signal both in controls' and patients' fibroblasts. A closer image analysis supported by morphometric quantification displayed an elongated interconnected mitochondrial network in control cell whereas unveiled a significantly more fragmented mitochondrial network in parkin1 fibroblasts (derived from the patient with severe clinical outcomes). Resveratrol treatment did not rescue in parkin1 the mitochondrial morphological alterations neither had significant effects on the mitochondrial morphology of the other cell samples.

4. Discussion

Parkin plays a pivotal role in the mitochondrial quality-control mechanisms [6] whereby a fine balance of mitochondrial autophagy and biogenesis is achieved [45]. In this work, it is shown that resveratrol treatment in *parkin*-mutated fibroblasts partially rescues the mitochondrial respiratory capacities via a pathway in which PGC-1α, master regulator of mitochondrial function, could be the ultimate recipient. Treatment of fibroblasts with resveratrol leads to a modest increase of basal and maximal respiration by endogenous substrates measured both in control and in *parkin*-mutant intact cells. Consistent with the resveratrol-mediated effect on respiration is the significant increase of CI activity. Noticeably, specific defects in CI biogenesis and/or in its electron transfer activity have long been recognized to be relevant in the development of parkinsonism [46]. The observed increase of CI activity by resveratrol treatment could be due to either mitochondrial biogenesis induction or regulation through post-translational modification or both. Previous studies indicate that activation of the cAMP/PKA pathway reverses the inhibition of the CI activity and the accumulation of ROS, effects associated with cAMP-dependent phosphorylation of the 18-kDa (AQDQ) subunit of CI [47,48]. An increase in mitochondrial biogenesis in PD's fibroblasts could be argued in view of the fact that resveratrol treatment resulted in an increase of citrate synthase activity and of mtDNA content.

The improvement of the mitochondrial respiratory activity induced by resveratrol treatment resulted in a significant increase in mitochondrial

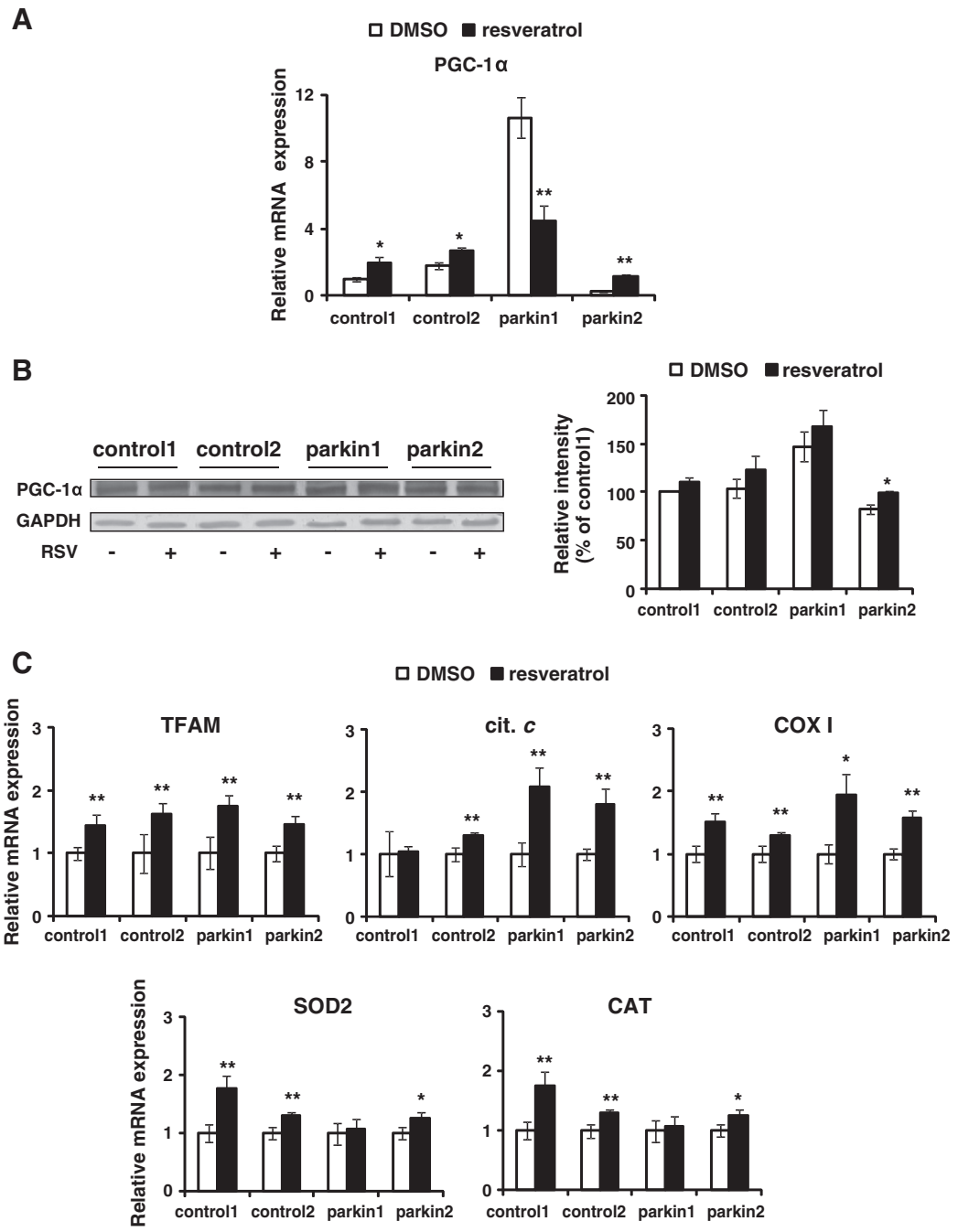

Fig. 9. Effect of resveratrol on expression and transcriptional activity of PGC-1α. (A) PGC-1α mRNA levels in controls' and patients' fibroblasts exposed to either vehicle (DMSO) or 25 μM resveratrol for 24 h. Values, determined by reverse transcriptase q-PCR analysis of total RNA, represent mRNA levels normalized to the housekeeping gene GAPDH. Relative expression values were compared with vehicle-treated control1. Data are means ± SEM and significance was calculated with Student's *t* test; n = 4 under each condition; *p < 0.05, **p < 0.005 vs vehicle-treated cells, °°p < 0.005, for parkin1 and parkin2 vs vehicle-treated control1. (B) Representative Western blot of PGC-1α performed on whole cell lysates from controls' and patients' fibroblasts exposed to either vehicle (DMSO) or 25 μM resveratrol for 24 h. The graph displays the statistical densitometric analysis of band intensity of PGC-1α normalized to the corresponding GAPDH level, used as loading control. Data, means ± SEM, are expressed as percentage of vehicle-treated control1; n = 4 under each condition. Significance was calculated with Student's *t* test; *p < 0.05 vs vehicle-treated cells, °p < 0.05, for parkin1 and parkin2 vs vehicle-treated control1. (C) mRNA levels of PGC-1α target genes: TFAM, cyt *c*, COXI, SOD2 and CAT in fibroblasts exposed to either vehicle (DMSO) or 25 μM resveratrol for 24 h. Values, determined by reverse transcriptase q-PCR analysis of total RNA, represent mRNA levels normalized to the housekeeping gene GAPDH. Relative expression values were compared with vehicle-treated cells. Data are means ± SEM and significance was calculated with Student's *t* test; n = 5 under each condition; *p < 0.05, **p < 0.005 vs vehicle-treated cells. For more details see Materials and methods.

ATP content in control as well as in patients' cells. In parkin1 and parkin2 fibroblasts this was associated with a significant decrease in lactate production and in a specific increase of citrate synthase, suggesting a switch from glycolysis to oxidative metabolism with an increased overall metabolic capacity. A similar effect of resveratrol has been %recently described by Miccheli's group [49] in a hepatocyte cell culture model with a switch from glucose and amino acid to fatty acid utilization for the energy production.

Resveratrol is a relatively strong activator of AMPK, a well-established sensor of low metabolic fuel in cell cultures and in animal model [16,50–53]. The precise mechanism by which resveratrol turns on AMPK is not firmly established as well as if the kinase activation is

or is not dependent on SIRT1 [51,54]. The earlier proposal of SIRT1 as a direct putative target of the resveratrol action [55] has been challenged based on lack of specificity in screening assays [56]. Very recently, Park et al. suggested that resveratrol is not directly targeting SIRT1 but instead stimulates the AMPK pathway through direct inhibition of cAMP-phosphodiesterases (mainly PDE4) and activation of the cAMP–Epac1–Camkkβ–AMPK signaling axis [57]. Anyway, PGC-1α activity has been shown to be necessary for AMPK-mediated mitochondrial biogenesis and function [37] as the phosphorylation of PGC-1α by AMPK is required for its subsequent SIRT1-mediated deacetylation [34]. Pharmacological (metformin) and physiological (fasting or exercise) activation of AMPK in muscle triggers an increase in the NAD^+/NADH ratio, which activates SIRT1. The impact of AMPK and SIRT1 on the phosphorylation/acetylation status of PGC-1α and other transcriptional regulators, leads to mitochondrial biogenesis and improved mitochondrial function [34]. We investigated, therefore, the possible involvement of the AMPK signaling activation in the beneficial effect of resveratrol. Whether resveratrol, in our conditions, also affects other pathways of PGC-1α activation needs to be further investigated.

Here we show that resveratrol causes, after 8 h of treatment, activation of AMPK and increase of the NAD^+/NADH ratio, which in turn would enable NAD^+-dependent SIRT1 activity as revealed by the decrease of the acetylated-H3. The NAD^+/NADH ratio is a dynamic measurement that reflects the metabolic activities of the cells and specific signaling pathways (PARPs, SIRT). At the specific time of incubation we measured the steady-state content of the oxidized and reduced forms of the nicotinamide nucleotides resulting by the overall processes that utilize and produce them. For this reason it is possible that we detected an increase of NADH/NAD^+ ratio at 8 h resulting from the increase of catabolic pathway induced by AMPK activation, values that subsequently go back to a new steady-state value resulting from the activity of specific cellular processes.

Since the main upstream AMPK-activating kinase is LKB1, whose activation requires the deacetylation activity of SIRT1 [54], we hypothesize that the activation of SIRT1 could also be responsible for LKB1 deacetylation. The activation of AMPK–SIRT1 signaling by resveratrol correlates with the induction of the PGC-1α activity, shown by the increased expression of its downstream target genes, and partly by the up-regulation of its expression. Albeit fully aware of the apparent incongruence of the conflicting data concerning PGC-1α in the two different patients' fibroblasts and the effect of resveratrol, nevertheless, we would like to highlight that PGC-1α activity is modulated both through the regulation of its expression and through the regulation of its activity by many posttranslational modifications, such as phosphorylation, acetylation, and ubiquitination, which enable it to fine-tune the activity of several transcription factors and the downstream pathways that they control [58]. Furthermore, resveratrol could also assist with the translocation of PGC-1α from the cytoplasm to the nucleus where PGC-1α may act as a regulator of mitochondrial biogenesis. All these possibilities warrant further investigations and are currently under scrutiny by our group. The paradoxical effect of resveratrol on PGC-1α mRNA level in parkin1 patient is in agreement with data from Farhoud et al. who described associated to the complex-I deficiency an enhanced PGC-1α signaling still achieved even when the gene expression of the co-activator protein is repressed [59].

Taken together, our findings suggest that resveratrol alleviates mitochondrial dysfunction in fibroblasts by coordinating signaling pathways in which AMPK is involved, in line with previous studies in cell line cultures [50–53]. This, in turn, results in an improvement of energy expenditure as well as in an enhancement of oxidative capacity attested by the increase in CI and citrate synthase activities, in basal oxygen consumption, in mitochondrial ATP production and by the decrease in lactate content. It should be stressed that the partial rescue of mitochondrial function could be due to the predominance of glycolytic metabolism in fibroblasts [60] with respect to oxidative metabolism typical of neurons [61].

It has to be taken into account that the interplay of the factors involved in the AMPK–SIRT1–PGC-1α axis and the downstream target genes of the latter does not make up in a linear sequence but rather

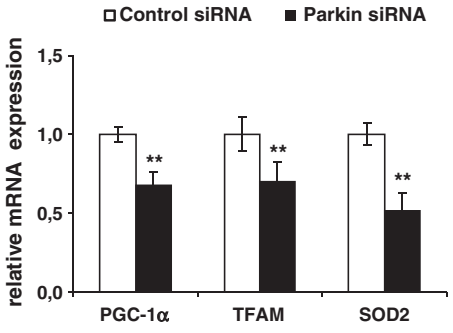

Fig. 10. mRNA expression levels of PGC-1α, TFAM and SOD2 in Control siRNA and Parkin siRNA fibroblasts. Values, determined by reverse transcriptase qRT-PCR analysis of total RNA represent mRNA levels normalized to the housekeeping gene GAPDH. Data are means ± SEM and significance was calculated with Student's *t* test, n = 3 under each condition; *p < 0.05, **p < 0.005 vs Control siRNA.

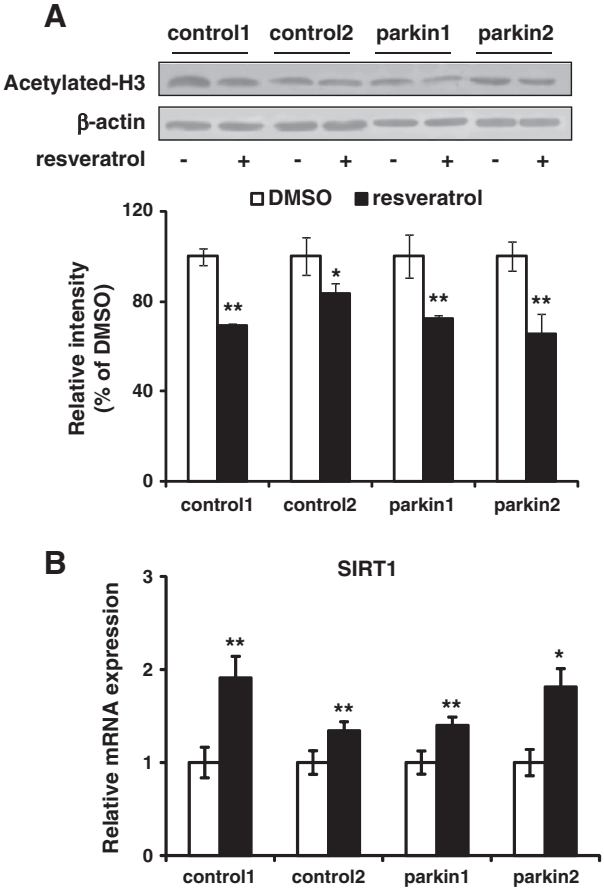

Fig. 11. Effect of resveratrol on SIRT1 activation. (A) Representative Western blot of acetylated-H3 performed on whole cell lysates from controls' and patients' fibroblasts exposed to either vehicle (DMSO) or 25 μM resveratrol for 8 h. The graph displays the densitometric analysis of band intensity of the acetylated H3 normalized to the corresponding β-actin level, used as loading control. Data, means ± SEM, are expressed as a percentage of vehicle-treated cells; n = 4 under each condition. Significance was calculated with Student's *t* test; *p < 0.05, **p < 0.005 vs vehicle-treated cells. (B) mRNA levels of SIRT1 in controls' and patients' fibroblasts exposed to either vehicle (DMSO) or 25 μM resveratrol for 24 h. Values, determined by reverse transcriptase q-PCR analysis of total RNA, represent mRNA levels normalized to the housekeeping gene GAPDH. Relative expression values were compared with vehicle-treated cells. Data are means ± SEM and significance was calculated with Student's *t* test; n = 4 under each condition;*p < 0.05, **p < 0.005 vs vehicle-treated cells. For more details see Materials and methods.

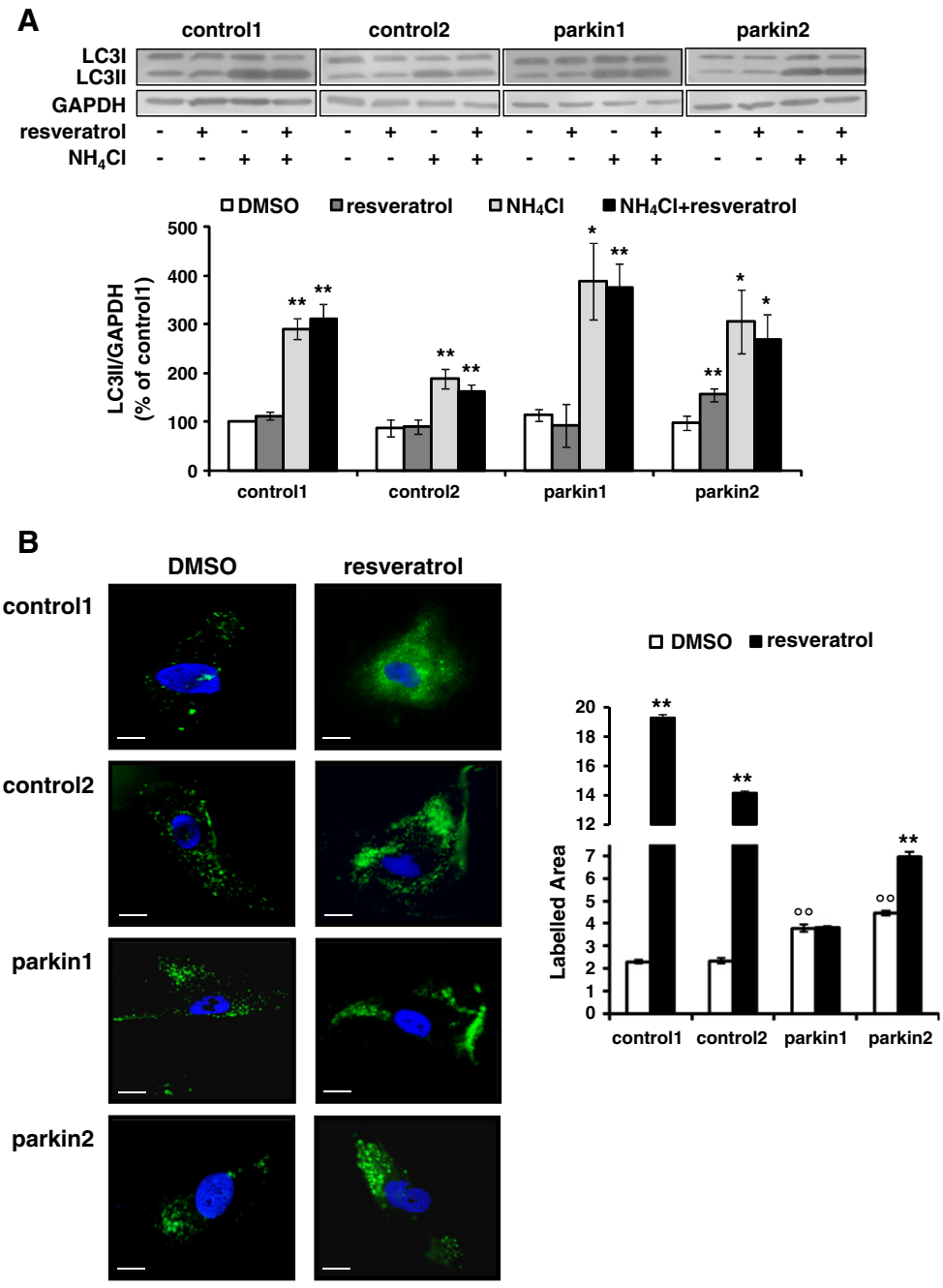

Fig. 12. Effect of resveratrol on autophagy induction. (A) Representative Western blot of LC3-I and LC3-II levels performed on whole cell lysates from controls' and patients' fibroblasts exposed for 24 h to either vehicle (DMSO) or 25 μM resveratrol, in the presence and in absence of 10 mM NH_4Cl. The graph displays the statistical analysis of the LC3-II content calculated by densitometric analysis of band intensity and normalized to the corresponding GAPDH level, used as loading control. Data, means ± SEM, are expressed as percentage of vehicle-treated control1; n = 4 under each condition. Significance was calculated with Student's *t* test; *p < 0.05, **p < 0.005 vs vehicle-treated cells. (B) Representative images of autophagic vacuoles detected by Cyto-ID® Autophagy Green dye in controls' and patients' fibroblasts, exposed to either vehicle (DMSO) or 25 μM resveratrol. Scale Bar: 15.8 μm. The graph displays the statistical analysis of the relative fluorescence intensity of cell labeled areas. Each bar is the mean ± S.D. from twenty fields from each experimental group; n = 3 under each condition. Significance was calculated with Student's *t* test; **p < 0.005 vs vehicle-treated cells; °°p < 0.005, for parkin1 and parkin2 vs vehicle-treated control1. For more details see Materials and methods.

comprises described feed-back mechanisms of reciprocal control. It is likely that in the PD derived fibroblasts the stringency of the control and the redundancy of other integrated signaling pathways are altered to make the cell surviving in the absence of parkin. Indeed, the observed shift toward a glycolysis-based metabolism in PD-fibroblasts represents the first line of adaptation aimed to relieve the cell from a defective quality-control of mitochondria. Consequently, it is not surprising that some of the effects of resveratrol result in abortive functional outcomes at least in the relatively short observational time-window of our treatment.

Moreover, recent results have showed that different dosages of resveratrol can elicit different responses. Price et al. [31] performed experiments using C2C12 cells in which high doses of resveratrol led to SIRT1-independent activation of AMPK and a decrease in NAD^+ and ATP levels, whereas lower doses of resveratrol led to SIRT1-dependent activation of AMPK and an increase in both metabolites.

Taken together, our findings suggest that the improvement of OXPHOS efficiency by resveratrol, through AMPK/SIRT1 pathway, could be related to a decrease of oxidative stress and to an increase of mitochondrial biogenesis. However, other mechanisms can be

envisaged to account for the observed resveratrol-mediated dramatic decrease of the pro-oxidative state in patients' cells. Among these are: i) direct inhibition of the ROS-generating system, likely related to the improved RC complex (specifically complex I) activity observed, ii) induction of antioxidant enzymes [62], and iii) intrinsic antioxidant properties [63]. All of these possibilities are not mutually exclusive and require further investigation.

The counter-intuitive observation linking reduced respiratory and complex I activities to increased mitochondrial ROS production in both patients' samples can be explained assuming the occurrence of subsets of the complex I population functionally impaired in transferring electrons downstream the respiratory chain. This would result in enhanced leakage of electrons (by the dysfunctional subset) and an overall decrease of the CI activity and of respiration. Moreover, it has to be taken in account that complex I itself is particularly vulnerable to oxidative insults thereby if the ROS-scavenging and/or the quality control system are not adequately functioning a vicious cycle establishes.

It is widely reported that nutritional antioxidants like resveratrol, carnosic acid, sulforaphane, dimethyl fumarate, acetyl-L-carnitine are able to activate vitagenes, such as heme oxygenase, Hsp70, thioredoxin reductase and sirtuins, an integrated system for cellular stress tolerance representing an innovative approach to therapeutic intervention in neurodegenerative disease [64–66].

AMPK activation induces inhibition of mTOR pathway, a major controller of protein homeostasis, through the autophagy and the ubiquitin–proteasome system [67]. Recently, Vingtdeux et al. reported that AMPK activation by resveratrol resulted in activation of autophagy and lysosomal clearance of amyloid β peptide [53]. We have investigated the autophagic process in our cell systems and found in the absence of changes of the LC3-II autophagy marker a significantly higher content of macroautophagic vesicles in both patients' fibroblast. After resveratrol treatment a many-fold induction of phagosomes was observed in control fibroblasts expressing parkin. Conversely, in parkin-deficient patients' fibroblasts resveratrol treatment caused no or limited effects on the phagosome content in parkin1 and parkin2 cells, respectively, consistent with their LC3-II profile. This result would imply the involvement of non-canonical alternative macroautophagic pathways as an adaptive response to parkin deficiency in patients' fibroblasts as well as their sensitivity to resveratrol but specifically in a parkin-dependent context.

Our study suggests a possible strategy to sustain and enhance mitochondrial functions by up-regulating key regulatory enzymes involved in metabolism and efficiency of mitochondrial bioenergetics [18,19]. Related to PD, resveratrol administration was shown to protect mice from MPTP-induced motor coordination impairment, hydroxyl radical overloading, and neuronal loss [22]. More recently, resveratrol has also been tested to provide beneficial effects in the 6-OHDA-induced PD rat model [24–26].

The impact of resveratrol treatment on the physiological activity of healthy cells is noteworthy. Indeed, although resveratrol does not appear, as expected, to rescue the lack of parkin in our mutant samples nevertheless our data suggest a disease-unrelated effect of the compound on mitochondrial function/biogenesis promoting mitochondrial “health” that tends to counteract the PD-mediated bioenergetic dysfunction. In our work we reported a strong effect induced by resveratrol treatment also in control fibroblasts in agreement with Csiszar's [68] and Bastin's [69] groups showing increased mitochondrial biogenesis in endothelial cells and enhanced fatty acid oxidation in normal fibroblasts, respectively, induced by resveratrol treatment. Furthermore, it should be pointed out that, the different responses to the resveratrol treatment between the two controls' and the two patients' cells could be attributed to the different genetic backgrounds and the large inter-individual variability.

5. Conclusion

Though evidences are emerging to support the potential of resveratrol against neurodegenerative disorders, no clear neuroprotective mechanism has been proposed so far. This study points to the SIRT1/AMPK/PGC1-α axis as a key neuroprotective pathway and provides a rationale for exploring the therapeutic potential of resveratrol in delaying PD progression at the initial stages of the disease or even before the onset of symptoms in its hereditary forms.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.02.010.

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References

[1] C. Pacelli, D. De Rasmo, A. Signorile, I. Grattagliano, G. di Tullio, A. D'Orazio, B. Nico, G.P. Comi, D. Ronchi, E. Ferranini, D. Pirolo, P. Seibel, S. Schubert, A. Gaballo, G. Villani, T. Cocco, Mitochondrial defect and PGC-1alpha dysfunction in parkin-associated familial Parkinson's disease, Biochim. Biophys. Acta 1812 (2011) 1041–1053.
[2] A. Grunewald, L. Voges, A. Rakovic, M. Kasten, H. Vandebona, C. Hemmelmann, K. Lohmann, S. Orolicki, A. Ramirez, A.H. Schapira, P.P. Pramstaller, C.M. Sue, C. Klein, Mutant Parkin impairs mitochondrial function and morphology in human fibroblasts, PLoS One 5 (2010) e12962.
[3] H. Mortiboys, K.J. Thomas, W.J. Koopman, S. Klaffke, P. Abou-Sleiman, S. Olpin, N.W. Wood, P.H. Willems, J.A. Smeitink, M.R. Cookson, O. Bandmann, Mitochondrial function and morphology are impaired in parkin-mutant fibroblasts, Ann. Neurol. 64 (2008) 555–565.
[4] H.H. Hoepken, S. Gispert, B. Morales, O. Wingerter, D. Del Turco, A. Mulsch, R.L. Nussbaum, K. Muller, S. Drose, U. Brandt, T. Deller, B. Wirth, A.P. Kudin, W.S. Kunz, G. Auburger, Mitochondrial dysfunction, peroxidation damage and changes in glutathione metabolism in PARK6, Neurobiol. Dis. 25 (2007) 401–411.
[5] P. del Hoyo, A. Garcia-Redondo, F. de Bustos, J.A. Molina, Y. Sayed, H. Alonso-Navarro, L. Caballero, J. Arenas, J.A. Agundez, F.J. Jimenez-Jimenez, Oxidative stress in skin fibroblasts cultures from patients with Parkinson's disease, BMC Neurol. 10 (2010) 95.
[6] D.P. Narendra, R.J. Youle, Targeting mitochondrial dysfunction: role for PINK1 and Parkin in mitochondrial quality control, Antioxid. Redox Signal. 14 (2011) 1929–1938.
[7] J.H. Shin, H.S. Ko, H. Kang, Y. Lee, Y.I. Lee, O. Pletinkova, J.C. Troconso, V.L. Dawson, T.M. Dawson, PARIS (ZNF746) repression of PGC-1alpha contributes to neurodegeneration in Parkinson's disease, Cell 144 (2011) 689–702.
[8] R.K. Chaturvedi, M.F. Beal, Mitochondrial approaches for neuroprotection, Ann. N. Y. Acad. Sci. 1147 (2008) 395–412.
[9] D.P. Kelly, R.C. Scarpulla, Transcriptional regulatory circuits controlling mitochondrial biogenesis and function, Genes Dev. 18 (2004) 357–368.
[10] B.N. Finck, D.P. Kelly, PGC-1 coactivators: inducible regulators of energy metabolism in health and disease, J. Clin. Invest. 116 (2006) 615–622.
[11] C. Handschin, B.M. Spiegelman, Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism, Endocr. Rev. 27 (2006) 728–735.
[12] B. Zheng, Z. Liao, J.J. Locascio, K.A. Lesniak, S.S. Roderick, M.L. Watt, A.C. Eklund, Y. Zhang-James, P.D. Kim, M.A. Hauser, E. Grunblatt, L.B. Moran, S.A. Mandel, P. Riederer, R.M. Miller, H.J. Federoff, U. Wullner, S. Papapetropoulos, M.B. Youdim, I. Cantuti-Castelvetri, A.B. Young, J.M. Vance, R.L. Davis, J.C. Hedreen, C.H. Adler, T.G. Beach, M.B. Graeber, F.A. Middleton, J.C. Rochet, C.R. Scherzer, PGC-1alpha, a potential therapeutic target for early intervention in Parkinson's disease, Sci. Transl. Med. 2 (2010) 52ra73.
[13] G. Mudo, J. Makela, V. Di Liberto, T.V. Tselykh, M. Olivieri, P. Piepponen, O. Eriksson, A. Malkia, A. Bonomo, M. Kairisalo, J.A. Aguirre, L. Korhonen, N. Belluardo, D. Lindholm, Transgenic expression and activation of PGC-1alpha protect dopaminergic neurons in the MPTP mouse model of Parkinson's disease, Cell. Mol. Life Sci. 69 (2012) 1153–1165.
[14] J. St-Pierre, S. Drori, M. Uldry, J.M. Silvaggi, J. Rhee, S. Jager, C. Handschin, K. Zheng, J. Lin, W. Yang, D.K. Simon, R. Bachoo, B.M. Spiegelman, Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators, Cell 127 (2006) 397–408.
[15] R.K. Chaturvedi, P. Adhihetty, S. Shukla, T. Hennessy, N. Calingasan, L. Yang, A. Starkov, M. Kiaei, M. Cannella, J. Sassone, A. Ciammola, F. Squitieri, M.F. Beal, Impaired PGC-1alpha function in muscle in Huntington's disease, Hum. Mol. Genet. 18 (2009) 3048–3065.
[16] J.A. Baur, K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu, J.S. Allard, G. Lopez-Lluch, K. Lewis, P.J. Pistell, S. Poosala, K.G. Becker, O. Boss, D. Gwinn, M. Wang, S. Ramaswamy, K.W. Fishbein, R.G. Spencer, E.G. Lakatta, D. Le Couteur, R.J. Shaw, P. Navas, P. Puigserver, D.K. Ingram, R. de Cabo, D.A. Sinclair, Resveratrol improves health and survival of mice on a high-calorie diet, Nature 444 (2006) 337–342.
[17] H.I. Rocha-Gonzalez, M. Ambriz-Tututi, V. Granados-Soto, Resveratrol: a natural compound with pharmacological potential in neurodegenerative diseases, CNS Neurosci. Ther. 14 (2008) 234–247.

[18] V. Foti Cuzzola, R. Ciurleo, S. Giacoppo, S. Marino, P. Bramanti, Role of resveratrol and its analogues in the treatment of neurodegenerative diseases: focus on recent discoveries, CNS Neurol. Disord. Drug Targets 10 (2011) 849–862.
[19] G.M. Pasinetti, J. Wang, P. Marambaud, M. Ferruzzi, P. Gregor, L.A. Knable, L. Ho, Neuroprotective and metabolic effects of resveratrol: therapeutic implications for Huntington's disease and other neurodegenerative disorders, Exp. Neurol. 232 (2011) 1–6.
[20] M. Lagouge, C. Argmann, Z. Gerhart-Hines, H. Meziane, C. Lerin, F. Daussin, N. Messadeq, J. Milne, P. Lambert, P. Elliott, B. Geny, M. Laakso, P. Puigserver, J. Auwerx, Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha, Cell 127 (2006) 1109–1122.
[21] M. Pallas, G. Casadesus, M.A. Smith, A. Coto-Montes, C. Pelegri, J. Vilaplana, A. Camins, Resveratrol and neurodegenerative diseases: activation of SIRT1 as the potential pathway towards neuroprotection, Curr. Neurovasc. Res. 6 (2009) 70–81.
[22] K.T. Lu, M.C. Ko, B.Y. Chen, J.C. Huang, C.W. Hsieh, M.C. Lee, R.Y. Chiou, B.S. Wung, C.H. Peng, Y.L. Yang, Neuroprotective effects of resveratrol on MPTP-induced neuron loss mediated by free radical scavenging, J. Agric. Food Chem. 56 (2008) 6910–6913.
[23] J. Blanchet, F. Longpre, G. Bureau, M. Morissette, T. DiPaolo, G. Bronchti, M.G. Martinoli, Resveratrol, a red wine polyphenol, protects dopaminergic neurons in MPTP-treated mice, Prog. Neuropsychopharmacol. Biol. Psychiatry 32 (2008) 1243–1250.
[24] M.M. Khan, A. Ahmad, T. Ishrat, M.B. Khan, M.N. Hoda, G. Khuwaja, S.S. Raza, A. Khan, H. Javed, K. Vaibhav, F. Islam, Resveratrol attenuates 6-hydroxydopamine-induced oxidative damage and dopamine depletion in rat model of Parkinson's disease, Brain Res. 1328 (2010) 139–151.
[25] Y. Wang, H. Xu, Q. Fu, R. Ma, J. Xiang, Protective effect of resveratrol derived from Polygonum cuspidatum and its liposomal form on nigral cells in parkinsonian rats, J. Neurol. Sci. 304 (2011) 29–34.
[26] F. Jin, Q. Wu, Y.F. Lu, Q.H. Gong, J.S. Shi, Neuroprotective effect of resveratrol on 6-OHDA-induced Parkinson's disease in rats, Eur. J. Pharmacol. 600 (2008) 78–82.
[27] M.K. Lee, S.J. Kang, M. Poncz, K.J. Song, K.S. Park, Resveratrol protects SH-SY5Y neuroblastoma cells from apoptosis induced by dopamine, Exp. Mol. Med. 39 (2007) 376–384.
[28] D. Albani, L. Polito, S. Batelli, S. De Mauro, C. Fracasso, G. Martelli, L. Colombo, C. Manzoni, M. Salmona, S. Caccia, A. Negro, G. Forloni, The SIRT1 activator resveratrol protects SK-N-BE cells from oxidative stress and against toxicity caused by alpha-synuclein or amyloid-beta (1–42) peptide, J. Neurochem. 110 (2009) 1445–1456.
[29] A. West, M. Periquet, S. Lincoln, C.B. Lucking, D. Nicholl, V. Bonifati, N. Rawal, T. Gasser, E. Lohmann, J.F. Deleuze, D. Maraganore, A. Levey, N. Wood, A. Durr, J. Hardy, A. Brice, M. Farrer, Complex relationship between Parkin mutations and Parkinson disease, Am. J. Med. Genet. 114 (2002) 584–591.
[30] G. Villani, G. Attardi, Polarographic assays of respiratory chain complex activity, Methods Cell Biol. 80 (2007) 121–133.
[31] N.L. Price, A.P. Gomes, A.J. Ling, F.V. Duarte, A. Martin-Montalvo, B.J. North, B. Agarwal, L. Ye, G. Ramadori, J.S. Teodoro, B.P. Hubbard, A.T. Varela, J.G. Davis, B. Varamini, A. Hafner, R. Moaddel, A.P. Rolo, R. Coppari, C.M. Palmeira, R. de Cabo, J.A. Baur, D.A. Sinclair, SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function, Cell Metab. 15 (2012) 675–690.
[32] J.H. Um, S.J. Park, H. Kang, S. Yang, M. Foretz, M.W. McBurney, M.K. Kim, B. Viollet, J.H. Chung, AMP-activated protein kinase-deficient mice are resistant to the metabolic effects of resveratrol, Diabetes 59 (2010) 554–563.
[33] H. Wei, Z. Zhang, A. Saha, S. Peng, G. Chandra, Z. Quezado, A.B. Mukherjee, Disruption of adaptive energy metabolism and elevated ribosomal p-S6K1 levels contribute to INCL pathogenesis: partial rescue by resveratrol, Hum. Mol. Genet. 20 (2011) 1111–1121.
[34] C. Canto, Z. Gerhart-Hines, J.N. Feige, M. Lagouge, L. Noriega, J.C. Milne, P.J. Elliott, P. Puigserver, J. Auwerx, AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity, Nature 458 (2009) 1056–1060.
[35] M. Fulco, Y. Cen, P. Zhao, E.P. Hoffman, M.W. McBurney, A.A. Sauve, V. Sartorelli, Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt, Dev. Cell 14 (2008) 661–673.
[36] C. Canto, J. Auwerx, AMP-activated protein kinase and its downstream transcriptional pathways, Cell. Mol. Life Sci. 67 (2010) 3407–3423.
[37] S. Jager, C. Handschin, J. St-Pierre, B.M. Spiegelman, AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 12017–12022.
[38] J. Wang, H. Fivecoat, L. Ho, Y. Pan, E. Ling, G.M. Pasinetti, The role of Sirt1: at the crossroad between promotion of longevity and protection against Alzheimer's disease neuropathology, Biochim. Biophys. Acta 1804 (2010) 1690–1694.
[39] A. Vaquero, M. Scher, D. Lee, H. Erdjument-Bromage, P. Tempst, D. Reinberg, Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin, Mol. Cell 16 (2004) 93–105.
[40] Y. Wu, X. Li, J.X. Zhu, W. Xie, W. Le, Z. Fan, J. Jankovic, T. Pan, Resveratrol-activated AMPK/SIRT1/autophagy in cellular models of Parkinson's disease, Neurosignals 19 (2011) 163–174.
[41] D. Narendra, A. Tanaka, D.F. Suen, R.J. Youle, Parkin is recruited selectively to impaired mitochondria and promotes their autophagy, J. Cell Biol. 183 (2008) 795–803.
[42] D. Chen, F. Gao, B. Li, H. Wang, Y. Xu, C. Zhu, G. Wang, Parkin mono-ubiquitinates Bcl-2 and regulates autophagy, J. Biol. Chem. 285 (2010) 38214–38223.
[43] D.J. Klionsky, et al., Guidelines for the use and interpretation of assays for monitoring autophagy, Autophagy 8 (2012) 445–544.
[44] K. Juenemann, E.A. Reits, Alternative macroautophagic pathways, Int. J. Cell Biol. 2012 (2012) 189794.
[45] R.A. Gottlieb, R.S. Carreira, Autophagy in health and disease. 5. Mitophagy as a way of life, Am. J. Physiol. Cell Physiol. 299 (2010) C203–C210.
[46] R.H. Swerdlow, J.K. Parks, S.W. Miller, J.B. Tuttle, P.A. Trimmer, J.P. Sheehan, J.P. Bennett Jr., R.E. Davis, W.D. Parker Jr., Origin and functional consequences of the complex I defect in Parkinson's disease, Ann. Neurol. 40 (1996) 663–671.
[47] F. Bellomo, C. Piccoli, T. Cocco, S. Scacco, F. Papa, A. Gaballo, D. Boffoli, A. Signorile, A. D'Aprile, R. Scrima, A.M. Sardanelli, N. Capitanio, S. Papa, Regulation by the cAMP cascade of oxygen free radical balance in mammalian cells, Antioxid. Redox Signal. 8 (2006) 495–502.
[48] D. De Rasmo, A. Signorile, M. Larizza, C. Pacelli, T. Cocco, S. Papa, Activation of the cAMP cascade in human fibroblast cultures rescues the activity of oxidatively damaged complex I, Free Radic. Biol. Med. 52 (2012) 757–764.
[49] M. Massimi, A. Tomassini, F. Sciubba, A.P. Sobolev, L.C. Devirgiliis, A. Miccheli, Effects of resveratrol on HepG2 cells as revealed by (1)H-NMR based metabolic profiling, Biochim. Biophys. Acta 1820 (2012) 1–8.
[50] C.E. Park, M.J. Kim, J.H. Lee, B.I. Min, H. Bae, W. Choe, S.S. Kim, J. Ha, Resveratrol stimulates glucose transport in C2C12 myotubes by activating AMP-activated protein kinase, Exp. Mol. Med. 39 (2007) 222–229.
[51] B. Dasgupta, J. Milbrandt, Resveratrol stimulates AMP kinase activity in neurons, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 7217–7222.
[52] J.T. Hwang, D.W. Kwak, S.K. Lin, H.M. Kim, Y.M. Kim, O.J. Park, Resveratrol induces apoptosis in chemoresistant cancer cells via modulation of AMPK signaling pathway, Ann. N. Y. Acad. Sci. 1095 (2007) 441–448.
[53] V. Vingtdeux, L. Giliberto, H. Zhao, P. Chandakkar, Q. Wu, J.E. Simon, E.M. Janle, J. Lobo, M.G. Ferruzzi, P. Davies, P. Marambaud, AMP-activated protein kinase signaling activation by resveratrol modulates amyloid-beta peptide metabolism, J. Biol. Chem. 285 (2010) 9100–9113.
[54] F. Lan, J.M. Cacicedo, N. Ruderman, Y. Ido, SIRT1 modulation of the acetylation status, cytosolic localization, and activity of LKB1. Possible role in AMP-activated protein kinase activation, J. Biol. Chem. 283 (2008) 27628–27635.
[55] K.T. Howitz, K.J. Bitterman, H.Y. Cohen, D.W. Lamming, S. Lavu, J.G. Wood, R.E. Zipkin, P. Chung, A. Kisielewski, L.L. Zhang, B. Scherer, D.A. Sinclair, Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan, Nature 425 (2003) 191–196.
[56] D. Beher, J. Wu, S. Cumine, K.W. Kim, S.C. Lu, L. Atangan, M. Wang, Resveratrol is not a direct activator of SIRT1 enzyme activity, Chem. Biol. Drug Des. 74 (2009) 619–624.
[57] S.J. Park, F. Ahmad, A. Philp, K. Baar, T. Williams, H. Luo, H. Ke, H. Rehmann, R. Taussig, A.L. Brown, M.K. Kim, M.A. Beaven, A.B. Burgin, V. Manganiello, J.H. Chung, Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases, Cell 148 (2012) 421–433.
[58] P.J. Fernandez-Marcos, J. Auwerx, Regulation of PGC-1alpha, a nodal regulator of mitochondrial biogenesis, Am. J. Clin. Nutr. 93 (2011) 884S–890S.
[59] M.H. Farhoud, L.G. Nijtmans, R.J. Wanders, H.J. Wessels, E. Lasonder, A.J. Janssen, R.R. Rodenburg, L.P. van den Heuvel, J.A. Smeitink, Impaired ubiquitin–proteasome-mediated PGC-1alpha protein turnover and induced mitochondrial biogenesis secondary to complex-I deficiency, Proteomics 12 (2012) 1349–1362.
[60] K. Brand, Aerobic glycolysis by proliferating cells: protection against oxidative stress at the expense of energy yield, J. Bioenerg. Biomembr. 29 (1997) 355–364.
[61] S.L. Mironov, Complexity of mitochondrial dynamics in neurons and its control by ADP produced during synaptic activity, Int. J. Biochem. Cell Biol. 41 (2009) 2005–2014.
[62] Z. Ungvari, Z. Orosz, A. Rivera, N. Labinskyy, Z. Xiangmin, S. Olson, A. Podlutsky, A. Csiszar, Resveratrol increases vascular oxidative stress resistance, Am. J. Physiol. Heart Circ. Physiol. 292 (2007) H2417–H2424.
[63] S.S. Leonard, C. Xia, B.H. Jiang, B. Stinefelt, H. Klandorf, G.K. Harris, X. Shi, Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses, Biochem. Biophys. Res. Commun. 309 (2003) 1017–1026.
[64] C. Cornelius, R. Perrotta, A. Graziano, E.J. Calabrese, V. Calabrese, Stress responses, vitagenes and hormesis as critical determinants in aging and longevity: Mitochondria as a "chi", Immun. Ageing 10 (2013) 15.
[65] E.J. Calabrese, M.P. Mattson, V. Calabrese, Dose response biology: the case of resveratrol, Hum. Exp. Toxicol. 29 (2010) 1034–1037.
[66] E.J. Calabrese, M.P. Mattson, V. Calabrese, Resveratrol commonly displays hormesis: occurrence and biomedical significance, Hum. Exp. Toxicol. 29 (2010) 980–1015.
[67] P. Polak, M.N. Hall, mTOR and the control of whole body metabolism, Curr. Opin. Cell Biol. 21 (2009) 209–218.
[68] A. Csiszar, N. Labinskyy, J.T. Pinto, P. Ballabh, H. Zhang, G. Losonczy, K. Pearson, R. de Cabo, P. Pacher, C. Zhang, Z. Ungvari, Resveratrol induces mitochondrial biogenesis in endothelial cells, Am. J. Physiol. Heart Circ. Physiol. 297 (2009) H13–H20.
[69] J. Bastin, A. Lopes-Costa, F. Djouadi, Exposure to resveratrol triggers pharmacological correction of fatty acid utilization in human fatty acid oxidation-deficient fibroblasts, Hum. Mol. Genet. 20 (2011) 2048–2057.