Biochimica et Biophysica Acta 1842 (2014) 902–915

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

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

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ARTICLE INFO

Article history:
Received 24 June 2013
Received in revised form 13 February 2014
Accepted 19 February 2014
Available online 25 February 2014

Keywords:
Parkinson's disease
Parkin
Mitochondria
Resveratrol
PGC-1α
Sirtuin 1

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.

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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http://dx.doi.org/10.1016/j.bbadis.2014.02.010
0925-4439/

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