




Metabolically induced intracellular pH changes activate mitophagy, autophagy, and cell protection in familial forms of Parkinson's disease

Nafisa R. Komilova², Plamena R. Angelova¹ , Alexey V. Berezhnov^{3,4}, Olga A. Stelmashchuk⁴, Ulugbek Z. Mirkhodjaev², Henry Houlden⁵, Alexander V. Gourine⁶, Noemi Esteras¹  and Andrey Y. Abramov^{1,4} 

1 Department of Clinical and Movement Neurosciences, UCL Queen Square Institute of Neurology, London, UK

2 Department of Biophysics, National University of Uzbekistan, Tashkent, Uzbekistan

3 Institute of Cell Biophysics of the Russian Academy of Sciences, Pushchino, Russia

4 Cell Physiology and Pathology Laboratory, Orel State University, Russia

5 Department of Neurodegenerative diseases, UCL Queen Square Institute of Neurology, London, UK

6 Department of Neuroscience, Physiology, Pharmacology, University College London, UK

Keywords

autophagy; lactate; mitophagy; Parkinson's disease; pyruvate

Correspondence

A. Y. Abramov, Department of Clinical and Movement Neurosciences, UCL Queen Square Institute of Neurology, Queen Square, London, WC1N 3BG, UK
 Tel: +44 2034484062
 E-mail: a.abramov@ucl.ac.uk

(Received 14 July 2021, revised 26 August 2021, accepted 13 September 2021)

doi:10.1111/febs.16198

Parkinson's disease (PD) is a progressive neurodegenerative disorder induced by the loss of dopaminergic neurons in midbrain. The mechanism of neurodegeneration is associated with aggregation of misfolded proteins, oxidative stress, and mitochondrial dysfunction. Considering this, the process of removal of unwanted organelles or proteins by autophagy is vitally important in neurons, and activation of these processes could be protective in PD. Short-time acidification of the cytosol can activate mitophagy and autophagy. Here, we used sodium pyruvate and sodium lactate to induce changes in intracellular pH in human fibroblasts with PD mutations (Pink1, Pink1/Park2, α -synuclein triplication, A53T). We have found that both lactate and pyruvate in millimolar concentrations can induce a short-time acidification of the cytosol in these cells. This induced activation of mitophagy and autophagy in control and PD fibroblasts and protected against cell death. Importantly, application of lactate to acute brain slices of WT and Pink1 KO mice also induced a reduction of pH in neurons and astrocytes that increased the level of mitophagy. Thus, acidification of the cytosol by compounds, which play an important role in cell metabolism, can also activate mitophagy and autophagy and protect cells in the familial form of PD.

Introduction

Parkinson's disease (PD) is a progressive, age-related currently incurable neurological disease characterized by tremor, rigidity, bradykinesia, and postural instability. The histopathological hallmark of PD is the loss of dopaminergic neurons in *substantia nigra* of the midbrain and the formation of intraneuronal or intragial inclusions of Lewy bodies consisting of aggregated α -synuclein. The

majority of PD cases are sporadic with about 10% of the discovered in the last decades being familial forms of this disorder [1]. Despite the strong progress in understanding the etiology of PD, the molecular and cellular mechanisms of neurodegeneration remain unclear.

Mitochondrial dysfunction is strongly implicated in the mechanism of sporadic (toxic) and familial forms

Abbreviations

ATP, adenosine triphosphate; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; KO, knockout; PD, Parkinson's disease; PINK1, PTEN-induced kinase 1; WT, wild-type.

of PD. Most proteins encoded by PD-related genes are connected to mitochondrial function. Altered adenosine triphosphate (ATP) production, overproduction of reactive oxygen species, and abnormal calcium handling in mitochondria are shown to be triggers for neuronal cell death in PD [2,3]. Additionally, damaged mitochondria might not be removed from neurons due to the alteration of the mechanism of mitochondrial quality control: the mitochondria-specific autophagic mechanism—mitophagy [4]. Parkinsonian genes are shown to be involved in the mechanism of mitophagy, specifically two of them—PINK1 and Parkin [5,6].

Autophagy is the process whereby intracellular components, including proteins and/or organelles, are delivered to lysosomes to be degraded. Autophagy maintains cellular homeostasis because it is the major contributor to basal turnover of cytosolic material, and a quality control system for proteins and organelles [7]. Processes of autophagy and mitophagy are also very important in PD due to the accumulation of α -synuclein toxic aggregates, which can target mitochondria and induce oxidative stress [8–10]. Considering this, activation of mitophagy and autophagy is suggested as a potential strategy for protection against misfolded aggregates and neurodegeneration in general [11]. There are a number of molecular pathways for mitophagy and autophagy and activation thereof can be beneficial and potentially neuroprotective for PD. Mitophagy and autophagy can be activated by different triggers including starvation and ischemic condition [12]. One of the major triggers for mitophagy used in experimental models is the application of high concentration of protonophores such as carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) or carbonyl cyanide m-chlorophenyl hydrazone (CCCP). We have suggested that these triggers may be involved in changes in intracellular pH and found that mitophagy and autophagy could also be activated by a short acidification of the cytosol [13]. However, the alternative inductor of pH decrease—nigericin—is toxic and cannot be used for neuronal protection.

Maintenance of intracellular pH is a sophisticated mechanism, which includes multiple enzymes and organelles, and importantly, cell metabolism [14,15]. Some metabolites, such as lactate, are believed to play an important role in brain energy metabolism and interaction between neurons and astrocytes [16,17]. Additionally, lactate concentration can dramatically increase during physical exercise [18] and it can induce angiogenesis and neurogenesis via lactate receptors [19]. Lactate is used by lactate dehydrogenase, which converts lactate to pyruvate. Both lactate and pyruvate can change intracellular pH and are metabolized inside

cells. Considering this, here we tested the dose-dependent effects of lactate and pyruvate on intracellular pH of patient's fibroblasts with various PD mutations. We also studied how these cytosolic pH changes influence mitophagy and cell viability.

Results

Basal intracellular pH is similar in control and in most Parkinson's patients' fibroblasts

To assess basal levels of cytosolic pH in control fibroblasts and patient's fibroblasts with familial forms of PD, we used SNARF-1 as a fluorescent indicator. We found that in cells with A53T, SNCA, PINK1, PINK1/Parkin mutations, SNCA triplication $[pH]_i$ was in the range of 7.1–7.2 (Fig. 1A). However, in patient cells with Phosphoglycerate kinase type 1 mutation (c.1132A>C, p. Thy378Pro) intracellular basal pH was significantly lower compared with control cells ($n = 40$ cells; $pH = 6.7 \pm 0.1$; $P < 0.05$; Fig. 1A).

Lactate dose-dependently reduce cytosolic pH in cells with familial forms of PD

Concentration of one of the major metabolites in the body, lactate, can dramatically change during physical activity and reach a concentration of up to 30 mM [20,21]. Application of sodium lactate (5–30 mM) to control fibroblasts dramatically reduced cytosolic pH in these cells (Fig. 1B,C). The minimal values were reached within 2–3 min with slow recovery of $[pH]_i$, and 10 mM NH_4Cl was then applied and washed out for calibration of the SNARF-1 signal (Fig. 1B). In control fibroblasts, the maximal decrease in pH was observed with a concentration of 10 mM lactate ($N = 3$; $n = 30$ cells; Fig. 1B,C). Effect of sodium lactate on the $[pH]_i$ of fibroblasts with PINK1 or PINK1/Parkin mutations was even bigger compared to control cells ($N = 3$; $n = 30$ cells; $P < 0.05$; Fig. 1D–F). Cells with α -synuclein pathology—SNCA triplication (Fig. 1G,H) or A53T mutation (Fig. 1I)—also had a significant reduction in pH in response to sodium lactate. Only one out of all studied cell lines—patient cells with phosphoglycerate kinase type 1 mutation (c.1132A>C, p. Thy378Pro)—had no or very moderate decrease of intracellular pH in response to 5–30 mM of sodium lactate (Fig. 1J,K). It should be noted that lowering of extracellular pH from 7.4 to 7.1 did not alter the effects of sodium lactate on intracellular pH of fibroblasts. Thus, application of sodium lactate can induce cytosolic acidification in control and fibroblasts from patients with familial forms of PD.

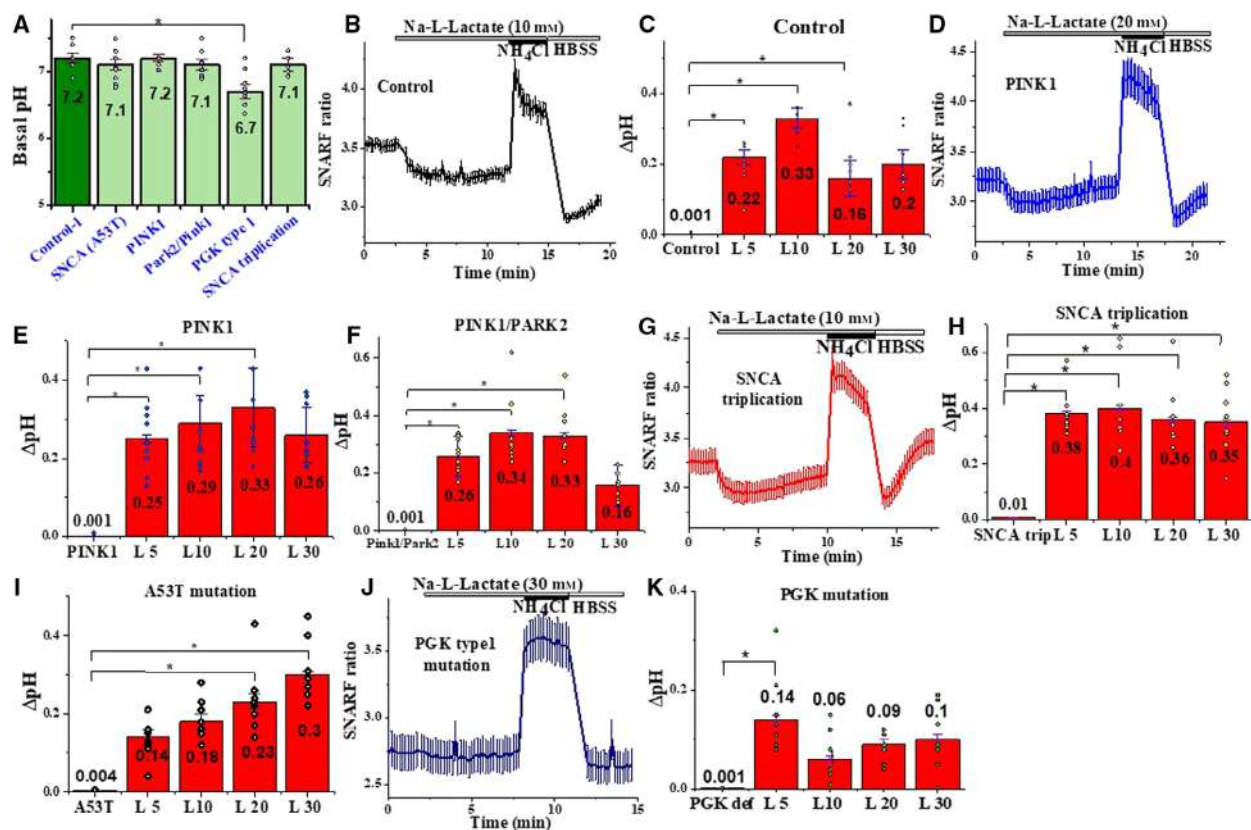


Fig. 1. Sodium lactate reduced intracellular pH in PD patients' fibroblasts. (A) basal [pH]_i in control fibroblasts and cells with PD-related mutations. (B, D, G, J) representative SNARF-1 ratio traces in control, PINK1 mutation, SNCA triplication, and PGK type 1 mutation fibroblasts in response to sodium lactate. NH₄Cl was added and washed out of medium for calibration of the SNARF-1 signal. Concentration-dependent changes of [pH]_i in response to sodium L-lactate (millimolar concentrations labeled as L5–L30 on the x-axis) in control (C), PINK1 mutation (E), PINK1/Park2 mutation (F), SNCA triplication (H), A53T mutation (I), and PGK type 1 mutation fibroblasts (K). Values are represented as mean ± SD. **P* < 0.05. Independent experiments (*n*) are indicated as dots in the scatter plot.

Pyruvate dose-dependently reduced cytosolic pH in cells with familial forms of PD

Application of sodium pyruvate (5–30 mM) to control fibroblasts induced a dose-dependent decrease of intracellular pH (Fig. 2A,B). Effect of sodium pyruvate on cells with familial forms of PD was mostly similar to the effects of lactate on pH of these cells. Thus, fibroblasts with PINK1 mutation showed a strong dose-dependence with the maximal decrease of [pH]_i in response to 30 mM sodium pyruvate (Fig. 2C), while in cells with PINK1/Parkin mutation, addition of 10–30 mM pyruvate induced similar acidification of the cytosol of fibroblasts (Fig. 2D,E). Application of pyruvate to fibroblasts with SNCA triplication had a profound effect on cytosolic pH in all range of concentrations (5–30 mM) with maximal reduction at 20 mM pyruvate (Fig. 2F,G). Fibroblasts with A53T mutations also significantly reduced pH in response to 5–30 mM sodium pyruvate with the maximal values at

30 mM (Fig. 2H). In the same way as in experiments with lactate, patient cells with phosphoglycerate kinase type 1 mutation (c.1132A>C, p. Thy378Pro) had only very moderate decrease of intracellular pH in response to 5–30 mM of sodium pyruvate (Fig. 2I–J). It should be noted that lowering of extracellular pH from 7.4 to 7.1 did not change the effects of sodium pyruvate on intracellular pH of fibroblasts. Thus, millimolar concentration of pyruvate induced short-time acidification of the cytosol in control and fibroblasts with familial forms of PD.

Lactate increased mitophagy in control and PD patient's fibroblasts

To assess the effect of various concentrations of lactate on mitophagy, we first pre-loaded the cells with MitoTracker Green and LysoTracker Red to stain mitochondria and lysosomes, respectively, treated cells with

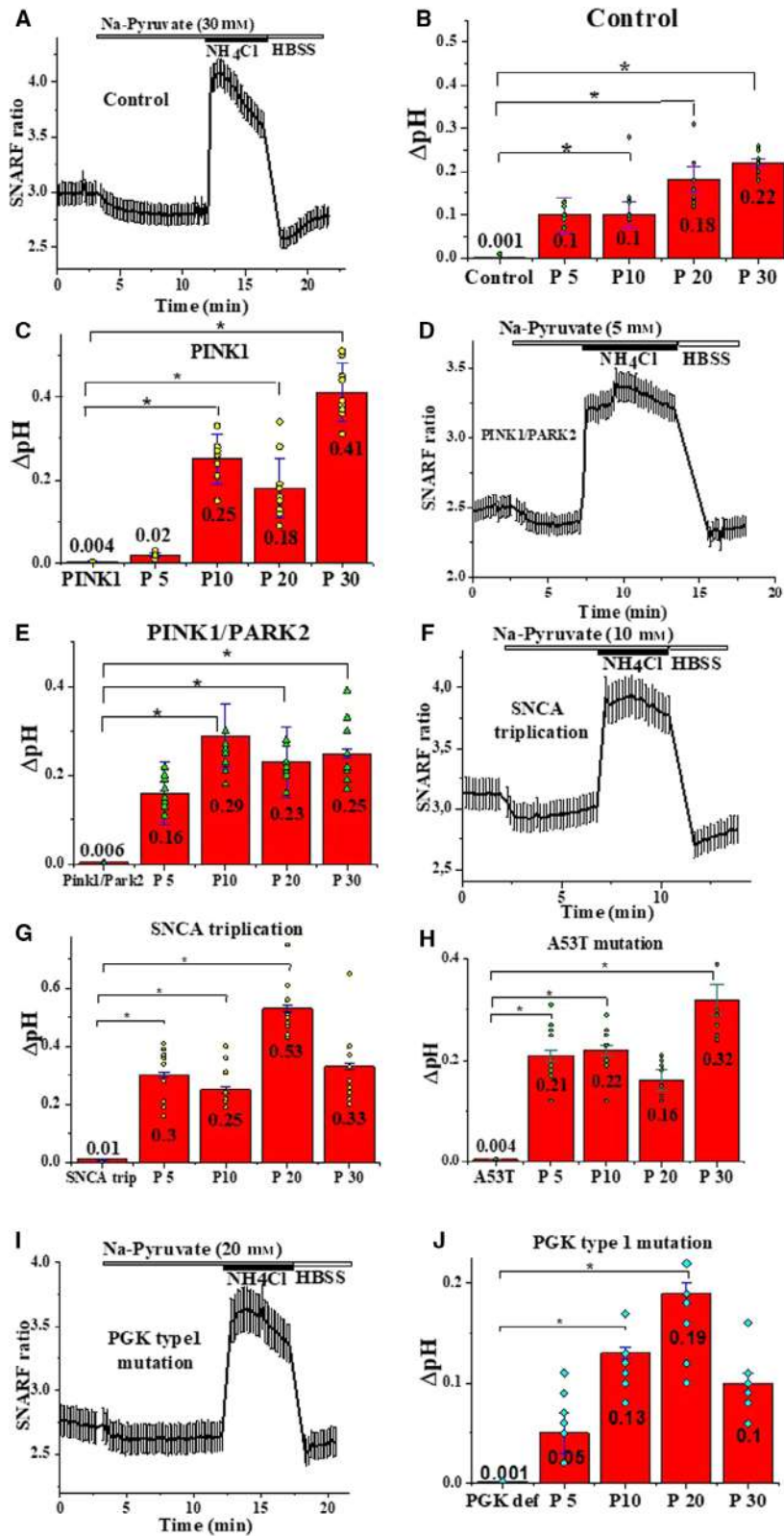


Fig. 2. Effect of sodium pyruvate on [pH]_i of PD patient's fibroblasts. (A, D, F, I) representative SNARF-1 ratio traces in control, PINK1/Park2 mutation, SNCA triplication, and PGK type 1 mutation fibroblasts in response to sodium pyruvate. NH₄Cl was added and washed out with HBSS for calibration of the SNARF-1 signal. Concentration-dependent changes of [pH]_i in response to sodium pyruvate (millimolar concentrations labeled as P5-P30 on x-axis) in control (B), Pink1 mutations (C), Pink1/Park2 mutation (E), SNCA triplication (G), A53T mutation (H), and PGK type 1 mutation fibroblasts (J). Values are represented as mean ± SD. **P* < 0.05. Independent experiments (*n*) are indicated as dots in the scatter plot.

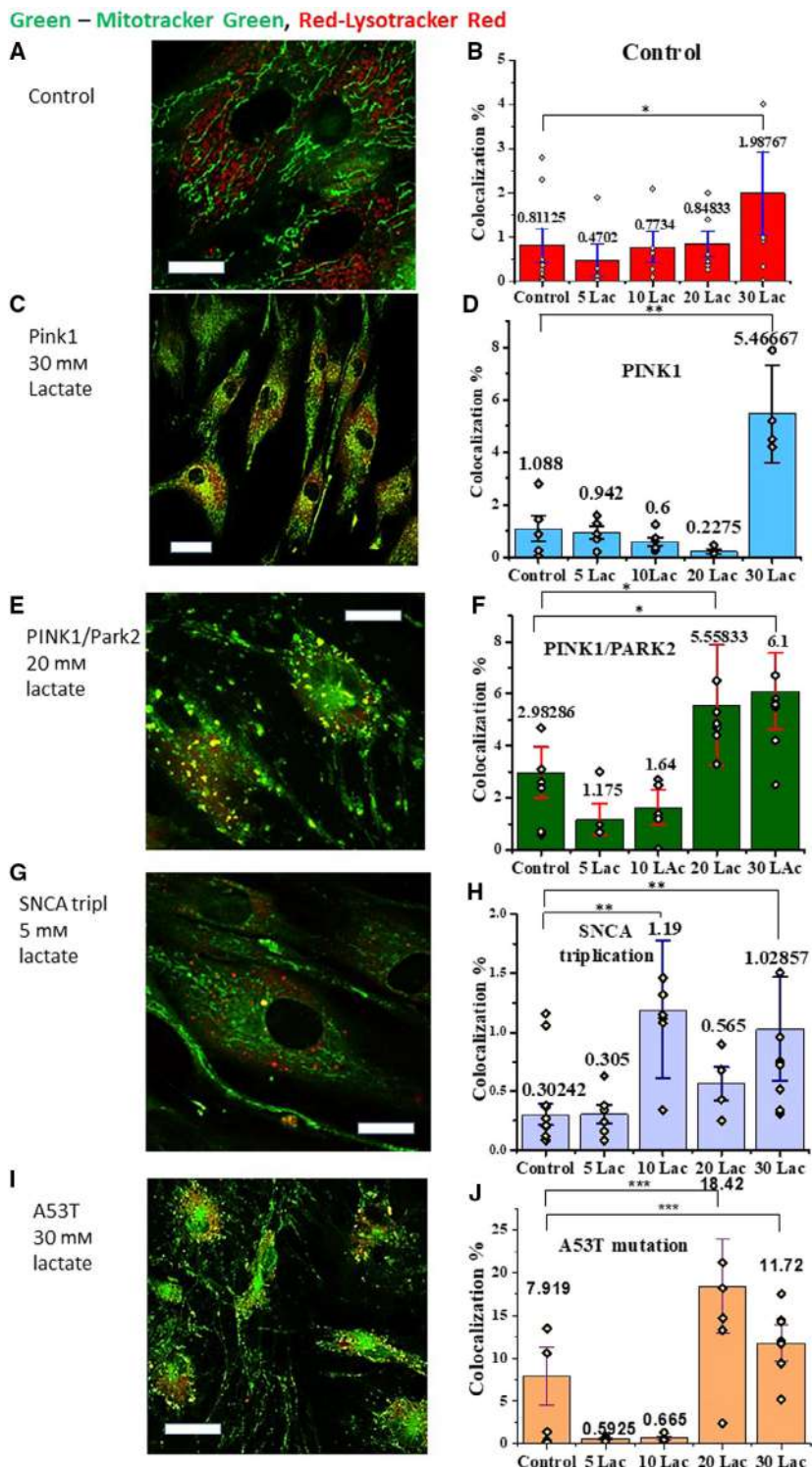


Fig. 3. Effect of sodium lactate on mitophagy in PD patient's fibroblasts. Concentration-dependent effect of sodium lactate on co-localization of mitochondria [labeled by MitoTracker Green (green color) and lysosomes] (LysoTracker Red, red color on images) in control fibroblasts (A, B), fibroblasts with PINK1 mutation (C, D), Pink1/Park2 mutation (E, F), α -synuclein (SNCA) triplication (G, H), and A53T mutation (I, J). Scale bars in images 20 μ m. Values are represented as mean \pm SD. * $P < 0.05$. Independent experiments (n) are indicated as dots in the scatter plot.

lactate for 2 h and, after imaging, calculated the percentage of mitochondria colocalizing with lysosomal signal.

The basal level of mitophagy in control fibroblasts was relatively low—only 0.81% ($N = 3$ experiments;

Fig. 3A,B). Incubation of the cells with lactate induced a significant increase in colocalization only at concentration of 30 mM ($N = 3$ experiments; $P < 0.05$; Fig. 3A,B).

Interestingly, despite Pink1 is implicated in the mechanism of mitophagy, fibroblasts with Pink1

mutation had a basal level of mitophagy comparable to control cells—1.08% ($N = 3$; Fig. 3C,D). 5–20 mM lactate even reduced the percentage of mitochondria colocalizing with lysosomes, but 30 mM lactate induced a significant 5-fold increase in the level of mitophagy in these cells ($N = 3$; to 5.46%; $P < 0.05$; Fig. 3C,D).

Fibroblasts with Pink1/Park2 mutation had a higher basal level of mitophagy—2.9% ($N = 3$; Fig. 3E,F). Lower concentrations of lactate—5–10 mM—reduced the percentage of mitochondria colocalizing with lysosomes (Fig. 3E,F) in the same way as in cells with Pink1 mutation. However, 20 mM and 30 mM of lactate significantly increased the level of mitophagy in fibroblasts with Pink1/Park2 mutations (Fig. 3E,F).

Interestingly, the different cells with distinct α -synuclein pathology had a different effect on the basal mitophagy level. Thus, fibroblasts with SNCA triplication had a lower level compared to control cells (0.30%, Fig. 3G,H), while in fibroblasts with the A53T mutation the percentage of colocalization was significantly higher—7.9% ($N = 3$; Fig. 3I–J). However, incubation of these cells with lactate increased the level of mitophagy in fibroblasts with both types of pathology—in SNCA triplication starting from 10 mM and higher (Fig. 3G,H) and in A53T—at concentrations 20 mM and 30 mM (Fig. 3I–J).

Thus, despite the difference in the basal levels of mitophagy, all studied fibroblasts had a significantly higher colocalization of mitochondria with lysosomes in response to higher concentrations of lactate (20–30 mM).

Pyruvate increased mitophagy in control and PD patients' fibroblasts

5–10 mM pyruvate is often used as a substrate for complex I-related respiration for activation of oxidative phosphorylation in cells [22]. Incubation of control fibroblasts with 5–30 mM sodium pyruvate induced a significant increase in the percentage of mitochondria colocalizing with lysosomes, starting from a concentration of 10 mM (from basal 0.81% to 1.85% for 10 mM, $N = 3$ experiments; $P < 0.05$; Fig. 4A,B). Sodium pyruvate could also activate mitophagy in Pink1 mutation or Pink1/Park2 mutation fibroblasts but only at concentration 30 mM (Fig. 4C–F). Thus, in cells with Pink1 mutation 30 mM pyruvate induced a 5-fold increase in colocalization ($N = 3$ experiments; Fig. 4C,D) in Pink1/Park2-deficient fibroblasts the percentage of mitophagy grew from 3.1% to 9.3% ($N = 3$; $P < 0.05$; Fig. 4E,F).

Interestingly, the same tendency as in experiments with Pink1- and Pink1/Park2-deficient fibroblasts—reduction of the percentage of colocalized mitochondria

with lysosomes with a small concentration of pyruvate and significant increase of mitophagy at 30 mM pyruvate—was observed in experiments with A53T mutation fibroblasts (Fig. 4G,H). It should be noted that the basal rate of mitophagy in these cells was much higher than in Pink1- or Pink1/Park2-deficient fibroblasts (7.9% compared to 1.08% and 3.14%, respectively; Fig. 4C,E,G).

In fibroblasts with α -synuclein triplication, incubation of cells with sodium pyruvate significantly increased the percentage of mitochondria colocalizing with lysosomes starting from 5 mM (Fig. 4I–J). Although pyruvate induced a 2- to 3-fold increase in mitophagy in these fibroblasts, the percentage of colocalization rose only to 1.32%, compared to basal 0.36%.

Thus, incubation of control PD patient fibroblasts with millimolar concentrations of sodium pyruvate increased the level of mitophagy in these cells.

Lactate-induced autophagy in control and fibroblasts with familial forms of PD

Autophagic flux was evaluated by assessing the lysosomal conversion of the autophagosome marker LC3 by immunoblotting [23]. Application of lactate (30 mM) induced an increase in the ratio LC3II/I, indicating a rise in the number of autophagosomes, that reached a peak after 30–60 min, followed by a decrease, which would indicate lysosomal degradation of the autophagosomes (Fig. 5A–C). To prove that the elevation in LC3II/I was due to an increase in the autophagic flux and not to a block, cells were treated with the V-ATPase inhibitor bafilomycin A1 (50 nM, 60 min), which inhibits lysosomal degradation. A further increase in LC3II/I ratio was observed in the presence of bafilomycin A1 (Fig. 5B,D), thus indicating that lactate activates the autophagic flux. Although no significant peak in LC3 levels was observed in PINK1 cells at the time-points studied, a significant increase in LC3II/I ratio clearly indicating activation of autophagy in PINK1-mutated fibroblasts by 30 mM lactate occurred after co-treatment with bafilomycin A1, suggesting the peak might have occurred at an earlier time-point. Thus, even a short application of 30 mM sodium lactate induces activation of autophagy in control fibroblasts and in fibroblasts with PD-related mutations.

Lactate-induced mitophagy in brain slices from WT and PINK1 KO mice

In order to identify whether acidification of the cytosol with lactate could induce mitophagy in brain cells, we

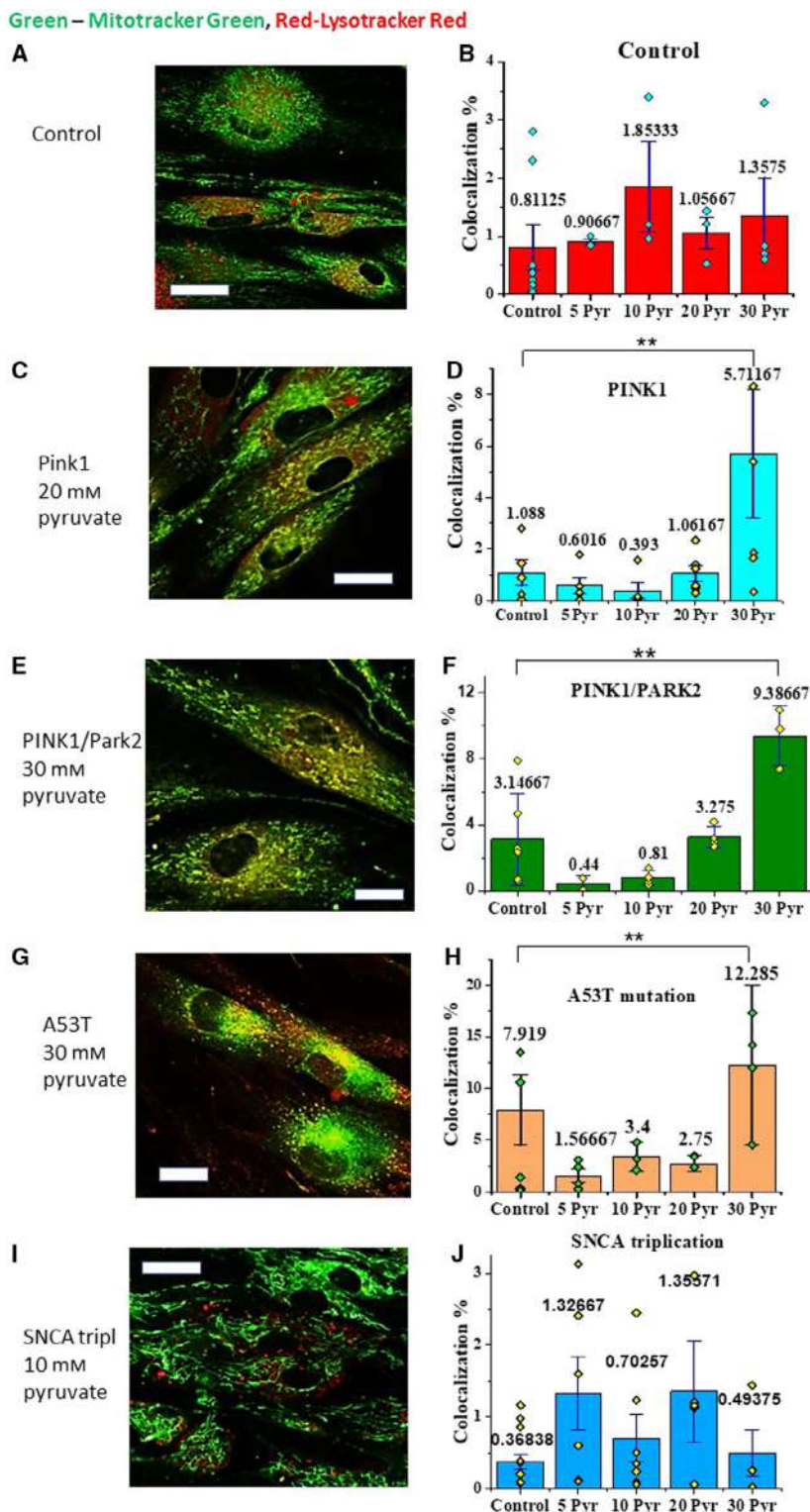


Fig. 4. Sodium pyruvate activated mitophagy in control and in fibroblasts with familial forms of PD. Concentration-dependent effect of sodium pyruvate on colocalization of mitochondria (labeled by MitoTracker Green (green color) and lysosomes (LysoTracker Red, red color on images) in control fibroblasts (A, B), fibroblasts with PINK1 mutation (C, D), Pink1/Park2 mutation (E, F), α -synuclein (SNCA) triplication (G, H), and A53T mutation (I, J). Scale bars in images 20 μ m. Values are represented as mean \pm SD. * P < 0.05. Independent experiments (n) are indicated as dots in the scatter plot.

used acute brain slices from adult wild-type (WT) and PINK1 knockout mice and a mitophagy detection kit. Interestingly, the basal level of mitophagy in PINK1

KO midbrain slices was 148.7% \pm 5.6% of WT slices (N = 5; Fig. 6A,B). 90-min incubation of the acute midbrain slices with 10 mM sodium L-lactate increased

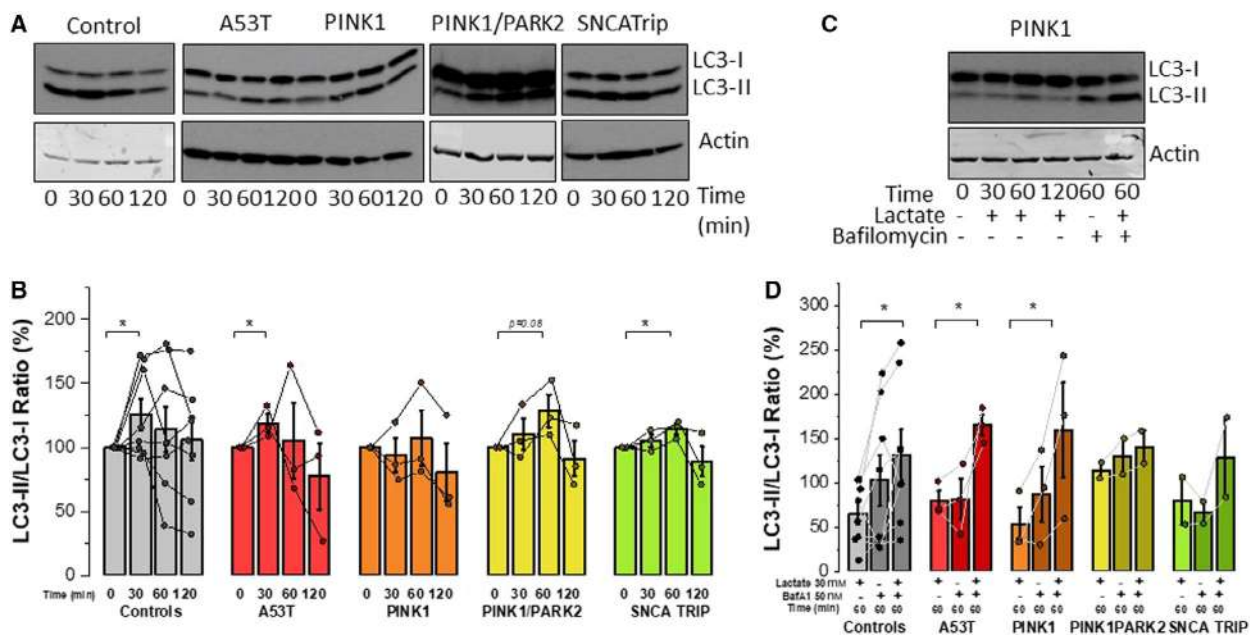


Fig. 5. Lactate increased autophagic flux in controls and PD patient's fibroblasts. (A, C) Representative immunoblots showing LC3 turnover after 30 mM sodium L-Lactate application. Increase in LC3-II correlates with autophagosome number. (B, D) Histograms represent LC3II/LC3I ratio at the different time-points/treatments indicated. Values are represented as mean \pm SD. Two sample *t*-test, **P* < 0.05. Independent experiments (*n*) are indicated as dots in the scatter plot.

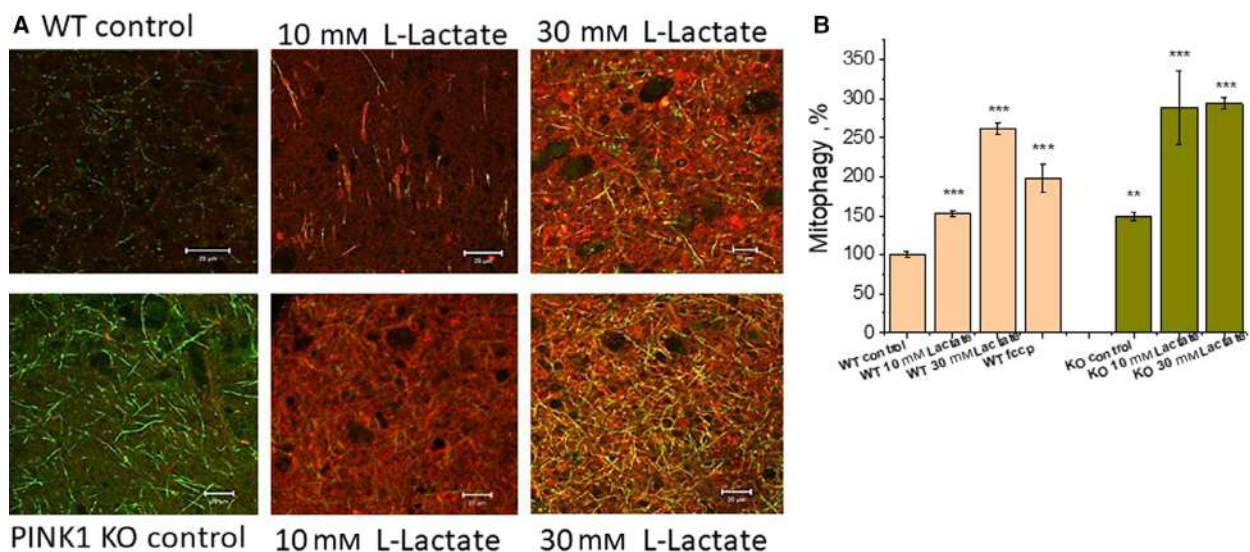


Fig. 6. Effect of sodium L-lactate on mitophagy in acute brain slices from WT and PINK1 KO mice. (A) representative images of mitophagy in acute brain slices from either WT (upper panels) or PINK1 KO (lower panels) mice (Mitophagy dye-red, Lyso dye-green). (B) Quantification bar-chart depict the percentage mitophagy induction in response to 10 mM or 30 mM sodium L-Lactate. WT brain slices were treated by protonophore, which is used in the most of works for activation of mitophagy—10 μ M FCCP as a positive control. Scale bars in images 20 μ m. Values are represented as mean \pm SEM. ****P* < 0.0001.

co-localization of mitochondria and lysosomes in cells of both WT and PINK1 KO brain slices (*N* = 5; Fig. 6A,B) inducing a 2-fold increase with higher

lactate concentration—30 mM compared to basal levels (*N* = 6; Fig. 6A-B). Importantly, this effect in WT slices was even higher than the effect of 10 μ M FCCP,

which is usually used for induction of mitophagy (Fig. 6B). Thus, incubation of the brain cells with sodium lactate can induce mitophagy in WT and PINK1 KO *ex vivo*.

Lactate and pyruvate reduced the number of dead cells in control fibroblasts and cells with familial forms of PD

Control fibroblasts and cells with PD mutations had ~ 6% dead cells in control conditions, with higher percentage in A53T mutation cell lines (9.8%, $N = 7$; Fig. 7A,B) and α -synuclein triplication (11.4%, $N = 9$; Fig. 7A,B). 24-h incubation of the cells with various concentrations of sodium lactate reduced the number of dead fibroblasts in the control cultures (to $3.3\% \pm 0.7\%$ at 20 mM; $N = 6$; $P < 0.05$; Fig. 7A). However, for cells with PINK1 mutation incubation with lactate was protective only in higher concentrations, 20 mM and 30 mM (to $3.2\% \pm 0.2\%$ and $2.2\% \pm 0.3\%$ for 20 mM and 30 mM; accordingly, $N = 6$; $P < 0.05$; Fig. 7A), and only at concentration of 30 mM for PINK1/Parkin mutation or fibroblasts with A53T mutation (Fig. 7A). Incubation of the fibroblasts with α -synuclein triplications with lactate effectively reduced the number of dead cells in the full range of concentrations from 5 mM to 30 mM (Fig. 7A). Thus, incubation of the patient's fibroblasts with millimolar concentrations of sodium lactate had no toxic effect on the cells and, in higher concentrations, was even protective.

24-h incubation of the fibroblasts with sodium pyruvate was protective for control cells, cells with A53T mutation, and α -synuclein triplications at concentrations from 5 mM to 30 mM (Fig. 7B). However, for cells with PINK1 mutation pyruvate protected cells from concentration 10 mM with maximal protection at 30 mM (to 2.1% dead cells; $N = 7$; $P < 0.001$; Fig. 7B). Surprisingly, 24-h incubation of fibroblasts with PINK1/Parkin mutation was protective only at lower concentrations (5–10 mM) but not at 20–30 mM (Fig. 7B). Thus, sodium pyruvate is protective for both control fibroblasts and for cells with PD mutations.

Discussion

Here, we have shown that two metabolites which participate in brain energy metabolism—pyruvate and lactate—can induce short-time concentration-dependent acidification of the cytosol. Importantly, these pH changes could be induced in cells with different genetic background. Effects of 30 mM lactate or pyruvate on intracellular pH of cells in the maximal value were

comparable to effects of brain ischemia, where $[pH]_i$ falls to 6.5 or below [24]. Changes in $[pH]_i$ regulate multiple processes in the cells. Thus, in neurons short-term acidification of the cytosol could decrease their neuronal activity [25], and in fibroblasts, low pH is shown to inhibit the process of cell dividing [26]. Although low pH is shown to be a trigger for apoptosis [27], in our experiment profound acidification (which was restored in our experiments within minutes) had only positive cell protective effect on control fibroblasts and cells with genetic form of PD (Fig. 7). However, considering the mitochondrial dysfunction in cells with these mutations [8,28,29] we cannot exclude effects of pyruvate and lactate on bioenergetics [30]. Additionally, in fibroblasts lactate can induce switch from oxidative phosphorylation to glycolysis that also potentially can have an effect of quality control of fibroblasts [31].

PD-related mutations induce neurodegeneration in specific brain areas, while neurons and astrocytes from different brain areas vary in their rate of energy metabolism and redox balance that may have an effect on mitophagy [32,33]. However, PD patients' fibroblasts are shown to be relevant models to study the effects of PD mutations on mitochondrial metabolism with results comparable to neuronal [29], and despite some limitations, using fibroblasts we can assess the effect of lactate and pyruvate on mitophagy and autophagy on cells with PD mutations.

Previously, we have shown that acidification of the cytosol with FCCP and nigericin induced mitophagy and PINK1-dependent and independent mitophagy and autophagy [13]. It should be noticed that changes in $[pH]_i$ in response to millimolar concentration of sodium lactate and sodium pyruvate were comparable with the effects of 10 μ M FCCP and 1–3 μ M nigericin, which induced mitophagy and autophagy. However, decrease in $[pH]_i$ in response to lactate and pyruvate did not induced cell death, which was observed with these ionophores.

PINK1- and Parkin-dependent mitophagy requires loss of mitochondrial membrane potential [34]. Lactate and pyruvate are mostly used to recover mitochondrial membrane potential [22,35], and considering this, the effect of lactate and pyruvate on mitophagy and autophagy is independent from mitochondrial membrane potential and induced by intracellular acidification. Importantly, decrease of $[pH]_i$ by lactate and pyruvate successfully induced mitophagy and autophagy in cells that lack PINK1 or PINK1/Parkin function that additionally confirms PINK1/Parkin-independent pathway of mitophagy, induced by millimolar concentrations of these compounds.

However, we cannot exclude this pathways' activation in cells with other mutations.

Levels of lactate in blood and intracellular acidification can be induced by a number of factors, which are shown to be beneficial, calorie restriction [36], and, importantly, physical exercise [37,38]. We can suggest that an elevation of the lactate level in the time of physical exercises of patients with PD could be potentially beneficial for activation of mitophagy and autophagy and consequently aiding neuroprotection.

Materials and Methods

Cell cultures

Fibroblasts were generated from a 4-mm skin punch biopsy taken under local anesthetic following local ethical approval. All patients gave full informed consent. We have ethical approval for investigating patients with informed consent and taking skin samples for research approved by University College London Hospital ethics committee (Number: 07/N018). Biopsies were dissected into ~1-mm pieces and cultured in DMEM, 10% FBS, and 1% GlutaMAX until fibroblasts were seen to grow out from the explants. When fibroblasts reached confluency, they were detached from culture dishes using TrypLE Express (Invitrogen) and transferred to larger culture vessels for further expansion and cryopreservation. Age, sex, clinical diagnosis, and gene mutations of the donors are depicted in Table 1.

Pyruvate and lactate applications

Sodium pyruvate and sodium lactate were dissolved in buffered HBSS at neutral pH (pH 7.4), and the applications of these compounds in all experiments were controlled for extracellular pH changes (all neutral).

Measurements of pH_{cyt}

Cells were loaded for 30 min at room temperature with 5 μ M 5-(and-6)-carboxyl SNARF-1 AM and 0.005% pluronic acid in a Hank's Balanced Salt Solution (HBSS) composed of (mM): 156 NaCl, 3 KCl, 1 MgSO₄, 1.25 KH₂PO₄, 2 CaCl₂, 10 glucose, and 10 HEPES; pH adjusted to 7.4 with NaOH. NH₄Cl was added and washed out to calibrate SNARF ratio. Confocal images were obtained using a Zeiss 710 confocal microscope equipped with 40 \times oil immersion objective. SNARF fluorescence was excited at 514 nm with argon laser light. Fluorescence was collected at 550–600 and 650–700 nm for ratiometric [pH]_c measurement every 10 s. SNARF-1 signal was calibrated according to the manufacturer's instructions.

Acute brain slices

Animals – PINK1-deficient mice, originally generated by Lexicon Genetics Inc. (The Woodlands, TX, USA), were obtained under MTA through Julian Downward, CRUK, London. Mice were maintained on a 12-h light/12-h dark cycle with free access to water and food. Animal husbandry and experimental procedures were performed in full

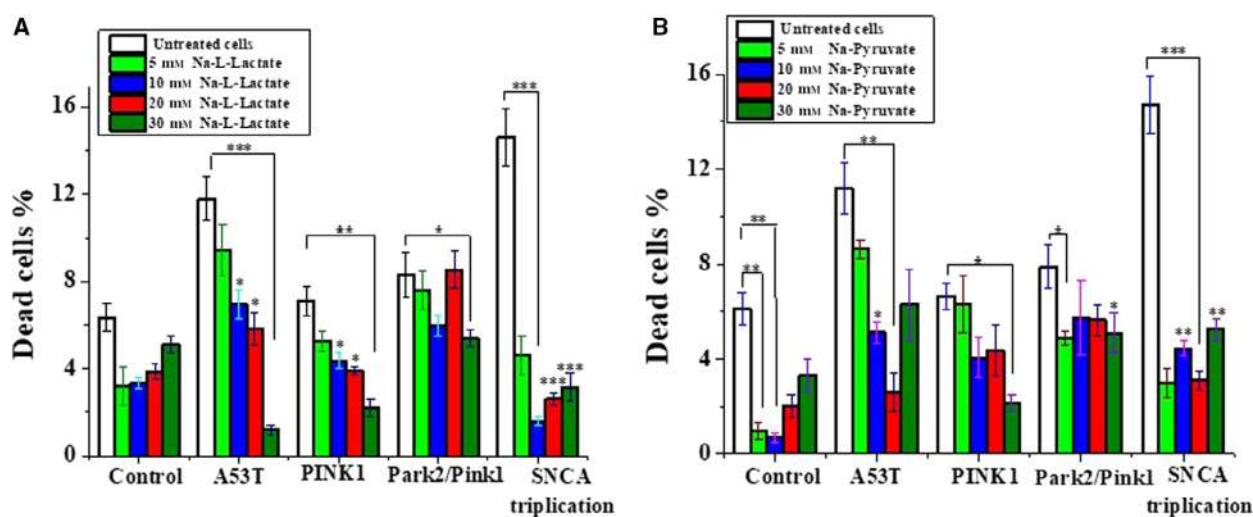


Fig. 7. Millimolar concentrations of sodium lactate and sodium pyruvate protect control and fibroblasts with familial PD against cell death. (A) Percentage of dead cells (labeled with PI) in control, and fibroblasts PINK1 mutation, PINK1/PARK2 mutations, A53T mutation and SNCA triplication after 24 h treatment with 5-, 10-, 20- or 30 mM sodium L-lactate. $N = 7$; (B) Percentage of dead cells in cultures of human fibroblasts treated with 5-, 10-, 20-, or 30-mM sodium pyruvate for 24 h. $N = 7$. Values are represented as mean \pm SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 1. Donors' information.

Line	Mutation	Diagnosis	Age	Sex
A53T	A53T het in SNCA	Severe PD	51	Female
PINK1	homozygous p. Try90Leufsx12 in PINK1	PD	52	Female
PINK1/Park2	Park2/Pink1 double heterozygous PARK2 R275W/WT + PINK1p. Try90Leu fs*12/WT	PD	75	Male
SNCA trip	Triplication in SNCA	Early-onset PD	55	Female
PGK-1	Phosphoglycerate kinase type 1 mutation (c.1132A>C,p. Thy378Pro)			
Control 1		Healthy donor	56	Female
Control 2		Healthy donor	49	Female
Control 3		Healthy donor	53	Male

compliance with the United Kingdom Animal (Scientific Procedures) Act of 1986 and with approval of the University College London Animal Ethics Committee. Animals were genotyped by PCR amplification of ear biopsy material in the following manner: genomic DNA was isolated from biopsy tissue using the Extract-N-Amp Tissue PCR kit (Merck, Burlington, MA, USA) and amplified with primers PINK1-26 (5'-CTGCCCTCAGGGTCTCTAATGC-3'), PINK1-27 (5'-GGAAGGAGGCCATGGAAATTGT-3'), and Neo (5'-GCAGCGCATCGCCTTCTATC-3'). PCR conditions were as follows: 94 °C denaturation—45 s, 60 °C annealing—45 s, and 72 °C extension—45 s. This cycle was repeated 30 times [39].

After extraction, the mice brains were placed in chilled HBSS. Transverse sections of midbrain with a thickness of 100 µm were cut, according to standard procedures [40,41] and stored prior to experiments for at least 1 h/37 °C.

Western blots

Protein extracts were collected in ice-cold RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Thermo Fisher, Paisley, UK). Samples were snap-frozen, sonicated, and centrifuged at 22 000 g, and protein content of the extracts was determined by the Pierce™ BCA protein assay (Thermo Fisher, Paisley, UK).

15–20 µg of protein was then fractionated on an SDS polyacrylamide gel (4%–12%) (Thermo Fisher, Paisley, UK), transferred to an Immobilon-P PVDF membrane (Merck), and blocked with 5% non-fat milk. Membranes were incubated overnight with the corresponding primary antibodies diluted in 5% bovine albumin serum: LC3 1 : 1000, p62 1 : 1000 or beta-actin (4970) 1 : 5000, all from Cell Signaling Technologies (Danvers, MA, USA) and afterward with the corresponding specie-specific HRP-conjugated secondary antibodies. The luminol-based Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) was used to detect the HRP activity. Protein band densities were quantified using IMAGEJ (NIH, Bethesda, MD, USA) after scanning the X-ray films. In order to compare the results between experiments, in all cases results were normalized to $t = 0$.

Mitochondria and lysosomes colocalization

For the identification of mitochondrial and lysosomal colocalization, cells were loaded with 200 nM MitoTracker Green FM and 50 nM LysoTracker Red DND-99 in HBSS for 30 min before experiments (including the treatment of cells with lactate and pyruvate).

The 488-nm Argon laser line was used to excite MitoTracker Green FM fluorescence, which was measured between 505 and 530 nm. Illumination intensity was kept to a minimum (about 1% of laser output) to avoid phototoxicity and the pinhole set to give an optical slice of ~ 2 µm. For LysoTracker Red DND-99, the 543 nm Ne/He laser line was used with measurement above 650 nm. All data presented were obtained from at least 5 coverslips and 2–3 different cell preparations.

Mitophagy detection kit

Mitophagy Detection Kit (Dojindo Molecular Technologies Inc., Kumamoto, Japan) is composed of a reagent for the detection of mitophagy (Mtphagy dye), and a reagent for labeling of the lysosomes (Lyso dye) was used to assess the levels of mitophagy. When mitophagy is induced upon certain treatment, the Mtphagy dye from damaged mitochondria increases in fluorescence and damaged mitochondria fuse to the lysosomes.

In brief, acute slices from WT and PINK1 KO mice were incubated with the Mtphagy dye (100 nmol·L⁻¹) and consequently treated with different concentrations of Lactate (10 mM, 30 mM) or FCCP (10 µM). After incubation period of 30 min, Mtphagy dye was washed and slices were incubated with the Lyso dye (1 µmol·L⁻¹) for 30 min. Slices were then washed and imaged with Zeiss 710 inverted confocal microscope (Mtphagy Dye, Ex: 561 nm/Em: 650; Lyso dye, Ex:488/Em:550 nm), correspondingly.

Cell death

Sodium lactate or sodium pyruvate in concentrations 5, 10, 20, or 30 mM or the same volume of buffer as a control were applied to the extracellular medium of the fibroblasts

and incubated for 24 h. Cells were then loaded for 15 min with 20 μM propidium iodide and 10 μM Hoechst 33342. While Hoechst is a blue, fluorescent dye that stains chromatin DNA, propidium iodide is only permeable to dead cells and shows red fluorescence, so it is possible to calculate the percentage of dead cells (showing red fluorescence) versus total number of cells (showing blue fluorescence). Fluorescence measurements were obtained on an epifluorescence-inverted microscope equipped with a 20 \times fluorite objective. Excitation light [for Hoechst 33342 380 nm and for PI (530 nm)] provided by a Xenon arc lamp. Emitted fluorescence light was reflected through a 515-nm long-pass filter to a cooled CCD camera (Retiga, QImaging, Canada). A total number of 800–1000 cells were counted in 4–5 different fields per coverslip. Experiments were repeated 4–7 times with separate independent cultures.

Statistical analysis

Results are expressed as means \pm SEM (standard error of the mean); one-way ANOVA with *post-hoc* Tukey's HSD correction for multiple comparisons and Student's *t*-test was used, where appropriate. Statistical analysis was performed using Origin 2019 (Microcal Software Inc., Northampton, MA, USA) software. Differences were considered to be significantly different if $P < 0.05$.

Acknowledgements

The study was supported by the EPSRC grant (Grant No. EP/R024898/1), by grant of the Russian Federation Government no. 075-15-2019-1877, and by the Russian Foundation for Basic Research (RFBR), project number 20-34-70074.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

AYA, AVB, AVG, NE, and PA involved in conceptualization. NK, PA, NE, OS, and AVB investigated and analyzed the data. AYA, HH, AVG, and UZM performed resources. AYA, NK, NE, and PA wrote original draft. AYA, NK, NE, PA, and AVB reviewed and edited the data. UZM, HH, OS, AVB, and AYA contributed to funding acquisition.

References

- Singleton A & Hardy J (2019) Progress in the genetic analysis of Parkinson's disease. *Hum Mol Genet* **28**, R215–R218.
- Ludtmann MHR & Abramov AY (2018) Mitochondrial calcium imbalance in Parkinson's disease. *Neurosci Lett* **663**, 86–90.
- Angelova PR & Abramov AY (2018) Role of mitochondrial ROS in the brain: from physiology to neurodegeneration. *FEBS Lett* **592**, 692–702.
- Lemasters JJ (2014) Variants of mitochondrial autophagy: types 1 and 2 mitophagy and micromitophagy (Type 3). *Redox Biol* **2**, 749–754.
- Pickrell AM & Youle RJ (2015) The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. *Neuron* **85**, 257–273.
- Beilina A & Cookson MR (2016) Genes associated with Parkinson's disease: regulation of autophagy and beyond. *J Neurochem* **139** (Suppl 1), 91–107.
- Hou X, Watzlawik JO, Fiesel FC & Springer W (2020) Autophagy in Parkinson's Disease. *J Mol Biol* **432**, 2651–2672.
- Abramov AY, Berezhnov AV, Fedotova EI, Zinchenko VP & Dolgacheva LP (2017) Interaction of misfolded proteins and mitochondria in neurodegenerative disorders. *Biochem Soc Trans* **45**, 1025–1033.
- Deas E, Cremades N, Angelova PR, Ludtmann MHR, Yao Z, Chen S, Horrocks MH, Banushi B, Little D, Devine MJ *et al.* (2016) Alpha-Synuclein oligomers interact with metal ions to induce oxidative stress and neuronal death in Parkinson's disease. *Antioxid Redox Signal* **24**, 376–391.
- Angelova PR, Esteras N & Abramov AY (2021) Mitochondria and lipid peroxidation in the mechanism of neurodegeneration: finding ways for prevention. *Med Res Rev* **41**, 770–784.
- Fang EF, Hou Y, Palikaras K, Adriaanse BA, Kerr JS, Yang B, Lautrup S, Hasan-Olive MM, Caponio D, Dan X *et al.* (2019) Mitophagy inhibits amyloid- β and tau pathology and reverses cognitive deficits in models of Alzheimer's disease. *Nat Neurosci* **22**, 401–412.
- Georgakopoulos ND, Wells G & Campanella M (2017) The pharmacological regulation of cellular mitophagy. *Nat Chem Biol* **13**, 136–146.
- Berezhnov AV, Soutar MPM, Fedotova EI, Frolova MS, Plun-Favreau H, Zinchenko VP & Abramov AY (2016) Intracellular pH modulates autophagy and mitophagy. *J Biol Chem* **291**, 8701–8708.
- Brookes N (1997) Intracellular pH as a regulatory signal in astrocyte metabolism. *Glia* **21**, 64–73.
- Chesler M (2003) Regulation and modulation of pH in the brain. *Physiol Rev* **83**, 1183–1221.
- Tang F, Lane S, Korsak A, Paton JF, Gourine AV, Kasparov S, & Teschemacher AG (2014) Lactate-mediated glia-neuronal signalling in the mammalian brain. *Nat Commun* **5**, 3284.
- Magistretti PJ & Allaman I (2018) Lactate in the brain: from metabolic end-product to signalling molecule. *Nat Rev Neurosci* **19**, 235–249.

- 18 Broskey NT, Zou K, Dohm GL & Houmard JA (2020) Plasma lactate as a marker for metabolic health. *Exerc Sport Sci Rev* **48**, 119–124.
- 19 Morland C, Andersson KA, Haugen ØP, Hadzic A, Kleppa L, Gille A, Rinholm JE, Palibrk V, Diget EH, Kennedy LH *et al.* (2017) Exercise induces cerebral VEGF and angiogenesis via the lactate receptor HCAR1. *Nat Commun* **8**, 15557.
- 20 Cheatham ME, Boobis LH, Brooks S & Williams C (1986) Human muscle metabolism during sprint running. *J Appl Physiol* **61**, 54–60.
- 21 Henderson GC, Horning MA, Wallis GA & Brooks GA (2007) Pyruvate metabolism in working human skeletal muscle. *Am J Physiol Endocrinol Metab* **292**, E366.
- 22 Abramov AY & Angelova PR (2019) Cellular mechanisms of complex I-associated pathology. *Biochem Soc Trans* **47**, 1963–1969.
- 23 Tanida I, Ueno T & Kominami E (2008) LC3 and Autophagy. In *Autophagosome and Phagosome* (Deretic V, ed), pp. 77–88. Humana Press, Totowa, NJ.
- 24 Rehncrona S, Rosen I & Smith ML (1985) Effect of different degrees of brain ischemia and tissue lactic acidosis on the short-term recovery of neurophysiologic and metabolic variables. *Exp Neurol* **87**, 458–473.
- 25 Sinning A & Hubner CA (2013) Minireview: pH and synaptic transmission. *FEBS Lett* **587**, 1923–1928.
- 26 Paris S & Pouyssegur J (1984) Growth factors activate the Na⁺/H⁺ antiporter in quiescent fibroblasts by increasing its affinity for intracellular H⁺. *J Biol Chem* **259**, 10989–10994.
- 27 Schwartz L, Peres S, Jolicoeur M & da Veiga Moreira J (2020) Cancer and Alzheimer's disease: intracellular pH scales the metabolic disorders. *Biogerontology* **21**, 683–694.
- 28 Gandhi S, Wood-Kaczmar A, Yao Z, Plun-Favreau H, Deas E, Klupsch K, Downward J, Latchman DS, Tabrizi SJ, Wood NW *et al.* (2009) PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. *Mol Cell* **33**, 627–638.
- 29 Abramov AY, Gegg M, Grunewald A, Wood NW, Klein C & Schapira AHV (2011) Bioenergetic consequences of PINK1 mutations in Parkinson disease. *PLoS ONE* **6**, e25622.
- 30 Gandhi S, Vaarmann A, Yao Z, Duchon MR, Wood NW & Abramov AY (2012) Dopamine induced neurodegeneration in a PINK1 model of Parkinson's disease. *PLoS ONE* **7**, e37564.
- 31 Kozlov AM, Lone A, Betts DH & Cumming RC (2020) Lactate preconditioning promotes a HIF-1 α -mediated metabolic shift from OXPHOS to glycolysis in normal human diploid fibroblasts. *Sci Rep* **10**, 8388.
- 32 Cheng XP, Vinokurov AY, Zhrebtsov EA, Stelmashchuk OA, Angelova PR, Esteras N & Abramov AY (2021) Variability of mitochondrial energy balance across brain regions. *Journal of Neurochemistry* **157**, 1234–1243.
- 33 Vinokurov AY, Stelmashuk OA, Ukolova PA, Zhrebtsov EA & Abramov AY (2021) Brain region specificity in reactive oxygen species production and maintenance of redox balance. *Free Radic Biol Med* **174**, 195–201.
- 34 Narendra DP & Youle RJ (2011) Targeting mitochondrial dysfunction: role for PINK1 and Parkin in mitochondrial quality control. *Antioxid Redox Signal* **14**, 1929–1938.
- 35 Erdelyi P, Borsos E, Takács-Vellai K, Kovács T, Kovács AL, Sigmond T, Hargitai B, Pásztor L, Sengupta T & Dengg M *et al.* (2011) Shared developmental roles and transcriptional control of autophagy and apoptosis in *Caenorhabditis elegans*. *J Cell Sci* **124** (Pt 9), 1510–1518.
- 36 Dutta D, Xu J, Dirain MLS & Leeuwenburgh C (2014) Calorie restriction combined with resveratrol induces autophagy and protects 26-month-old rat hearts from doxorubicin-induced toxicity. *Free Radic Biol Med* **74**, 252–262.
- 37 Volianitis S, Secher NH & Quistorff B (2018) Elevated arterial lactate delays recovery of intracellular muscle pH after exercise. *Eur J Appl Physiol* **118**, 2429–2434.
- 38 Vainshtein A, Tryon LD, Pauly M & Hood DA *et al.* (2015) Role of PGC-1 α during acute exercise-induced autophagy and mitophagy in skeletal muscle. *Am J Physiol Cell Physiol* **308**, C710–C719.
- 39 Deas E, Piipari K, Machhada A, Li A, Gutierrez-del-Arroyo A, Withers DJ, Wood NW & Abramov AY (2014) PINK1 deficiency in beta-cells increases basal insulin secretion and improves glucose tolerance in mice. *Open Biol* **4**, 140051.
- 40 Angelova P & Muller W (2006) Oxidative modulation of the transient potassium current IA by intracellular arachidonic acid in rat CA1 pyramidal neurons. *Eur J Neurosci* **23**, 2375–2384.
- 41 Novikova IN, Manole A, Zhrebtsov EA, Stavtsev DD, Vukolova MN, Dunaev AV, Angelova PR & Abramov AY (2020) Adrenaline induces calcium signal in astrocytes and vasoconstriction via activation of monoamine oxidase. *Free Radic Biol Med* **159**, 15–22.