Title:Exosome Release Is Modulated by the Mitochondrial-Lysosomal Crosstalk in Parkinson's Disease Stress Conditions

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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra (SN) pars compacta region of the brain. The main pathological hallmark involves cytoplasmic inclusions of α- synuclein and mitochondrial dysfunction, which is observed in other part of the central nervous system other than SN suggesting the spread of pathogenesis to bystander neurons. The inter-neuronal communication through exosomes may play an important role in the spread of the disease; however, the mechanisms are not well elucidated. Mitochondria and its role in inter-organellar crosstalk with multivesicular body (MVB) and lysosome and its role in modulation of exosome release in PD is not well understood. In the current study, we investigated the mitochondria-lysosome crosstalk modulating the exosome release in neuronal and glial cells. We observed that PD stress showed enhanced release of exosomes in dopaminergic neurons

and glial cells. The PD stress condition in these cells showed fragmented network and mitochondrial dysfunction which further leads to functional deficit of lysosomes and hence inhibition of autophagy flux. Neuronal and glial cells treated with rapamycin showed enhanced autophagy and inhibited the exosomal release. The results here suggest that maintenance of mitochondrial function is important for the lysosomal function and hence exosomal release which is important for the pathogenesis of PD.

Keywords Mitochondrial dysfunctions · Mitochondria-lysosome crosstalk · Exosome release · Parkinson's disease

Introduction

Parkinson's disease (PD) is a chronic, progressive, neurode- generative movement disorder including motor as well as non- motor symptoms. It affects 1% of the population of over 60 years of age and 3% of people over 80 years of age, and an estimated seven to ten million people are affected world- wide [1, 2]. Neuronal loss in the substantia nigra leads to a decrease in the dopamine levels in the corpus striatum, which leads to the motor symptoms, namely, tremor, rigidity and bradykinesia. The cellular hallmark of PD is the presence of the intracytoplasmic Lewy bodies and Lewy neurites, com- posed of protein aggregates, fats and polysaccharides. The protein aggregates contain α-synuclein, neurofilaments, ubiquitin, Parkin and Synphilin [3]. There are emerging evi- dences which show that misfolded proteins spread through the brain along anatomically connected networks to other neuro- nal regions thereby promoting progressive decline [4]. PD occurs sporadically as well as in a familial form. Mutations in genes like SNCA, LRRK2 and VPS35 are related to auto- somal dominant forms of PD, while PARKIN, PINK1 and DJ1 are associated with autosomal recessive form of PD [5]. The key molecular pathways regulated by these genes in- volved in PD are emerging; however, their role in progression to different brain regions is not well understood.

The dopaminergic neurons are specifically vulnerable in PD; however, α-synuclein aggregation and neuronal degener- ation are observed in non-dopaminergic parts of the brain as well, like neocortex, brain stem and olfactory bulb [6], sug- gesting that the pathology spreads to other types of brain cells including microglia and astrocytes and other parts of the brain. Emerging studies suggest that exosomes play a major role in inter-neuronal and neuron-glia communication in the brain [7]. Exosomes are a class of extracellular vesicles ranging in the size approximately 30–150 nm, which are released from almost all cell types including neuron, glial and astrocytes [8].

The exosomes are generated within multivesicular bodies (MVBs) that contain intra-luminal vesicles (ILVs) in the endosomal system. A variety of mRNAs, small non-coding RNAs, DNA and proteins, are selectively enriched into the ILVs of the MVBs, when the MVB fuses with the plasma membrane, ILVs in the form of exosomes are relased [9]. In PD-insulted neurons, α-synuclein is predominantly enriched in exosomes, transferred to nearby neurons lead- ing to neuronal dysfunction [10]. Further exosomes loaded with α-synuclein along with other components like miRNA, mRNA and DNA are uptaken by the glial cells and induce inflammation [11]. Overall, these evidences suggest that exosomes play a major role in inter-neuronal and neuronal-glial communication; however, the intracel- lular pathway regulating the exosomal release in neuronal and glial cells in the PD stress conditions are unclear.

Mitochondria is a dynamic organelle which has been im- plicated in various cellular functions including maintenance of bioenergetics by oxidative phosphorylation (OXPHOS), calcium homeostasis, apoptosis and inflammation [12]. Given its vital role in the cellular homeostasis and neuronal survival, mitochondrial dysfunction plays a central role in PD and other neurodegenerative diseases. The dysfunction is mainly characterized by a decrease in the complex I ac- tivity of the OXPHOS complex, generation of ROS (reac- tive oxygen species), ATP depletion and apoptosis. The turnover of dysfunctional mitochondria through selective process of autophagy called as mitophagy is important in neuronal survival including dopaminergic neurons [13]. Interestingly, genes involved in familial PD like PINK1 and PARKIN play essential role in turnover of mitochon- dria through mitophagy; hence, mutations in these genes lead to accumulation of damaged mitochondria [14, 15]. The effective turnover of dysfunctional mitochondria and alpha-syn aggregates requires efficient autophagy flux and in turn functional lysosomes. There are emerging studies which show that the autophagy-lysosome pathway is altered in PD [16]. Mutations in genes like ATP13A2/PARK9, cause of familial PD, also show impairment of lysosomal functions and accumulation of autophagosomes [17, 18]. LRRK2-mutant PD patient neurons (iPSCs) show altered macro autophagy and increased levels of α-synuclein in the cell

[19, 20]. These evidences suggest that autophagy is important for neuronal homeostasis; however, its role in exosome release and inter-neuronal communication is not understood.

Multivesicular body is known to play critical role in exosome biogenesis and recruitment of the misfolded protein and its degradation through the endo-lysosomal pathway [21]. MVBs fuse with the lysosome where their content is degraded and recycled back [22] and may also fuse with the plasma membrane, releasing the ILV content in the form of exosomes [23]. The mitochondria-lysosomal crosstalk is important for the process of autophagy and cellular homeostasis which in turn plays a role in exosomal release [24]. This crosstalk be- tween mitochondria, lysosome and exosome in neuronal cells are yet to be investigated systematically in PD stress condi- tions. In this study, we analysed the mitochondrial-lysosome crosstalk in PD stress conditions and its role in exosome re- lease from the neuronal cell in PD stress conditions.

Materials and Methods

Cell Culture and Reagents

SH-SY5Y, human neuroblastoma cells were grown at 37 °C, 5% CO_2 in Ham's F-12 Kaighn's Modification (F-12K, HyClone, GE Lifesciences) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Gibco, Invitrogen) and 1% (v/v) penicillin, streptomycin and neomy- cin (PSN) antibiotic mixture (Gibco, Invitrogen). U-87 MG and SK-N-SH cells were grown at 37 °C, 5% CO_2 in MEM/ EBSS (HyClone, GE Lifesciences) accompanied with 10% (v/v) heat-inactivated FBS, 1% (v/v) PSN and 1 mM sodium pyruvate (HyClone, GE Lifesciences). One-shot exosome-de- pleted FBS was obtained from Gibco, Invitrogen. All three cell lines were obtained from National Centre for Cell Sciences, Pune, India. mCherry-GFP-LC3 was provided by Dr. Terje Johansen (Dept. of

Biochemistry, Institute of Medical Biology, University of Tromsø). The primary anti- bodies used were anti-LC3 (Sigma-Aldrich, USA), anti-p62 (Cell Signalling, USA), anti-NDP52 (Cell Signalling, USA), anti-CD63 (Santa Cruz, USA), anti-actin (GenScript, USA), and anti-calnexin (Cell Signalling, USA). Secondary antibod- ies HRP-conjugated anti-rabbit and anti-mouse antibodies (Jackson ImmunoResearch, USA) were used. Rotenone, 6- OHDA, Bafilomycin A1, rapamycin and wortmannin were all purchased from Sigma-Aldrich, USA. For transfection, Lipofectamine® 3000 (Invitrogen, USA) was used. SH- SY5Y dopaminergic neuronal cells and U-87 MG glial cells were treated with 75 µM 6-OHDA and 0.01 µM Rotenone, whereas SK-N-SH dopaminergic neuronal cells were treated with 5 µM 6-OHDA and 0.1 µM Rotenone.

Exosome Isolation

Cells were seeded at a density of 4.5×10^5 cells per well in 6-well plate and were treated with pre-conditioned media containing exosome-depleted serum. The exosomes were isolated using an affinity purification-based method. The pre-conditioned media was collected and centrifuged at 2000 g for 30 min to settle down the cell debris. The supernatant was collected and Total Exosome Isolation reagent (Thermo Fisher Scientific, Invitrogen, USA) was added in 2:1 ratio volume. The mixture was incubated at 4 °C overnight. Following incubation, the mixture was centrifuged at 10,000 g for 1 h at 4 °C to obtain the exosome pellet. The exosome pellet was then used for either NTA or western blotting for further characterization.

Nanoparticle Tracking Analysis

Cells were seeded at a density of 2.5×10^5 cells per well in 12- well plate and were treated with the given

chemicals along with the pre-conditioned media. The pre-conditioned media was collected, and exosomes were isolated by the method described above. Exosome pellet was resuspended in 1 ml of DPBS solution and was analysed by the NanoSight NS300 (Malvern Panalytical, UK), which shows both the particle size as well as concentration.

Western Blotting

Cells were seeded at a density of 4.5×10^5 cells per well in 6-well plate and were treated with the given chemicals for the particular time period. After treatment, cells were har- vested, washed with ice cold PBS and lysed in NP40 lysis buffer (150 mM NaCl, 50 mM Tris–Cl, 5 mM EDTA, 1% NP40, 1% glycerol and 1× protease inhibitor cocktail (Sigma-Aldrich, USA). Protein concentration was deter- mined by Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad, USA), and equal amount of proteins were resolved on 12.5% SDS-PAGE. Proteins were electro-blotted on PVDF membrane (Immun-Blot® PVDF Membrane, Bio-Rad, USA) at 110 V for 1 h at 4 °C. Following the transfer, the membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) for 1 h at room temperature. The mem- brane was incubated overnight with specific primary anti- body. After incubation the membrane was washed three times with TBS-T (TBS containing 0.1% Tween-20) and incubated with a secondary antibody at room temperature for 1 h. The membrane was washed three times with TBS- T and signal visualized by using (Bio-Rad, USA) by ex- posing to X-ray film. The band intensity was quantified using ImageJ software and relative quantification was shown as compared to control samples.

Lysosomal Acid Phosphatase Assay

Cells were seeded at a density of 2.5×10^5 cells per well in 12- well plate and treated with the given

chemicals. After treat- ment, cells were collected and lysed with Passive Lysis Buffer (25 mM Tris-HCl, pH 7.8, 2 mM DTT, 2 mM EDTA, 10%

glycerol, 1% Triton X-100) for 30 min on ice. Protein concen- tration was determined by Bradford assay. Acid phosphatase activity was measured as a colorimetric assay. Ten micro- grams of lysate was incubated with 5 mM pNPP in 100 µl of citrate buffer (90 mM, pH 4.8) in 96-well plate for 30 min at 37 °C. The reaction was stopped with 100 mM NaOH solu- tion, and absorbance was measured at 405 nm in a microplate reader (Thermo Fisher Scientific, USA).

Fluorescence Microscopy

Cells were seeded at a density of 1.5×10^5 cells per well in 24- well plate and transfected with mCherry-GFP-LC3 construct and subsequently treated with given chemicals, 24 h post transfection. Fluorescence microscopy was performed using Eclipse Ti2-E inverted fluorescence microscope (Nikon, Japan) and analysed by ImageJ. Detectors gain, offset levels and laser power were calibrated at identical levels and remain unchanged for a set of experiment. Numbers and types (yel- low or red) of puncta per cell were counted in minimum 30 cells manually and graph plotted for the average number of LC3 puncta per cell.

Confocal Microscopy

For analysis of mitochondrial morphology, MT-RFP was transfected in cells and subsequently treated with the given chemicals, 24 h post transfection, and images were acquired using confocal microscope. All images were acquired with LSM 710 inverted confocal microscope (Carl Zeiss, Germany), and detectors gain, offset levels and laser power were calibrated at identical levels and remain unchanged for a set of experiment.

Similarly, cells were treated with the given chemicals for 24 h and stained with LysoTracker Blue (Invitrogen) post- treatment, and images were obtained using LSM 710 inverted confocal microscope, and detectors gain, offset levels and la- ser power were calibrated at identical levels and remain un- changed for a set of experiment. The number of lysosomes per cell was counted using the ITCN plugin in ImageJ software.

ATP Assay

The cellular ATP level was measured using ATP determina- tion kit (Molecular Probes/Life Technologies, Canada) by using 1:10 diluted cell lysate in ATP determination master mix (25 mM Tricine buffer, pH 7.8, 5 mM $MgSO_4$, 0.5 mM D-luciferin, 1.25 µg/ml firefly luciferase, 100 µM EDTA and 1 mM DTT). The luminescence intensity was measured using TriStar[2] LB 942 Multimode Microplate Reader, Berthold Technologies, Germany. The protein content was determined by Bradford assay and normalized with obtained intensity.

Spectrophotometric Analysis of Mitochondrial Complex I Assay

The activity of mitochondrial complex I was analysed spec- trophotometrically. Cells were seeded at the density of 9×10^5 cells in 60-mm dish. After treatment, cells were subjected to 2–3 freeze–thaw cycles in a freeze–thaw complete solution (0.25 M sucrose, 20 mM Tris-HCl (pH 7.4), 40 mM KCl, 2 mM EDTA supplemented with 1 mg/ml fatty acid-free BSA, 0.01% digitonin and 10% Percoll). The cells were washed again thrice with the freeze–thaw solution devoid of digitonin and resuspended in complex I assay buffer (35 mM potassium phosphate (pH 7.4), 1 mM EDTA,

2.5 mM NaN_3,1 mg/ml BSA, 2 µg/ml antimycin A, 5 mM NADH). The reaction was started by adding 80 µg of cell lysate to 500 µl of assay buffer in 1-ml quartz cuvette. Complex I activity was measured for

2 min by monitoring the decrease in absorbance at 340 nm after the addition of 2.5 mM acceptor decylubiquinone indicating the oxidation of NADH.

Statistical Analysis

Data are shown as mean ± SEM for number of times the experiment was repeated. Comparisons between two groups were performed using Student's t test for repeated measure- ments to determine the levels of significance for each group. The experiments were performed minimum two times inde- pendently and $p < 0.05$ was considered as statistically signifi- cant. GraphPad Prism (version 5) software was used to per- form all the statistical analysis.

Results

Exosome Release Is Enhanced in Parkinson's Disease Stress Conditions

The release of exosomes and its modulation in PD stress con- ditions in different neuronal cell types in brain is not well understood. Hence, we first isolated the exosomes from three different cell lines, including U-87 MG (glial cell origin), SH- SY5Y and SK-N-SH (neuronal cell line; expresses high levels of dopamine β hydroxylase) (Fig. 1a) and subsequently char- acterized the exosomes by Nanoparticle Tracking Analysis (NTA) and western blotting. The NTA analysis showed the particle size ranging from 80 to 150 nm which is the size range of exosomes (Fig. 1b (i)) in U-87 MG. Recent evidences show that the exosome population is rather heterogeneous in nature, based on which the exosomes are categorized into subpopu- lations of large exosome vesicles, Exo-L, 90–120 nm; small exosome vesicles, Exo-S, 60–80 nm; and exomeres, ~ 35 nm [25]. In line with these reports, the nanoparticle plots of all cell lines in PD stress

conditions also showed varying concentra- tions and population of different sizes, even including the microvesicles population (Supplementary Fig. S1). Western blot analysis shows bands of the size 30–60 KDa correspond- ing to CD-63, a well-characterized exosome marker in SK-N-SH dopaminergic neuronal cells. Calnexin (ER-resident protein) was used as a negative control for the exosome lysate. The absence of calnexin in the exosomal fraction further con- firmed its purity (Fig. 1b (ii)). 6-OHDA and Rotenone are widely used to generate experimental models of PD in vitro [26, 27]. We further analysed the release of exosomes from these cell lines in PD stress conditions like 6-OHDA and Rotenone. Acetylcholine esterase has been previously de- scribed to be enriched in exosomes [28] and so has been used as marker for exosomes. Hence, we analysed acetyl- choline esterase activity of the isolated exosomes in SH- SY5Y and SK-N-SH dopaminergic neuronal cells and U- 87 MG glial cells in different PD stress conditions, 6- OHDA and Rotenone, to analyse the quantitative release of exosomes. We observed enhanced level of acetylcholine esterase activity in exosomal fraction isolated from all cells in presence of 6-OHDA and Rotenone as compared to un- treated (Fig. 1c). We also used NTA analysis to quantify the release of the exosomes. Exosomes from cells treated with 6-OHDA and Rotenone subjected to NTA analysis showed increased concentration of exosomes in PD stress condi- tions in all the cell lines (Fig. 1d). Further, we analysed the levels of CD63 (a well-characterized exosome marker) in exosomes by western blotting in 6-OHDA and Rotenone conditions. Interestingly, increased level of exosomal CD63 was observed in 6-OHDA and Rotenone treated cells compared to control in all the cell lines (Fig. 1e). All the evidences provided here strongly suggest enhanced exosomal release in both neurons and glial cells in vitro in PD stress conditions.

Parkinson's Disease Stress Conditions Lead to Mitochondrial Dysfunctions

Mitochondrial dysfunctions affect a number of cellular path- ways leading to cell death; hence, we monitored mitochondri- al functions in PD stress conditions. We assessed the mito- chondrial morphology in PD stress conditions in U-87 MG glial cells by confocal microscopy. The untreated cells show an extensive mitochondrial network, as com- pared to the cells treated with 6-OHDA and Rotenone, which

show a fragmented mitochondrial network (Fig. 2a). Mitochondrial respiratory chain is the most important sites for ROS production, and alteration in com- plex I activity is one of the major ROS source as there are many electron transfer centres and possibilities of leakage of electron [29]. Hence, we analysed the complex I activity in PD stress conditions by spectrophotometric method in SH-SY5Y and SK-N-SH dopaminergic neuro- nal cells. Both SH-SY5Y and SK-N-SH showed a con- siderable decrease in the complex I activity in PD stress conditions (Fig. 2b). Complex I is the entry point of electrons from NADH in ETC (electron transport chain) in mitochondria and, hence, determines the level of ATP and ROS levels in cells. Therefore, we analysed the ATP levels in SH-SY5Y, SK-N-SH dopaminergic neuronal cells and U-87 MG glial cells. All the cell lines show a decrease in the ATP levels in the presence of 6-OHDA and Rotenone (Fig. 2c). These evidences clearly suggest that mitochondrial functions are altered in PD stress conditions.

Lysosomal Functions Are Altered in Parkinson's Disease Stress Conditions

The emerging evidences suggest that defect in OXPHOS ac- tivity leads to altered NADH/NAD ratio which also leads to lysosomal dysfunction [30]. This suggests that mitochondrial and lysosomal functions are linked [31]; however, this crosstalk between the lysosome and mitochondria is not well understood in PD; hence, we analysed the lysosomal functions in PD stress conditions. We assessed the lysosomes with LysoTracker Blue by confocal microscopy in the presence and absence of 6-OHDA and Rotenone in SH-SY5Y, SK-N- SH dopaminergic neuronal cells and U-87 MG glial cells. We observed significant decrease in the number of lysosomes, in all the three cell lines when treated with 6-OHDA and Rotenone (Fig. 3a).

The function of lysosomes is critical for autophagy and hence degradation of α-syn aggregates and turnover of mito- chondria; hence, we monitored the acid phosphatase activity of both neuronal and glial cells. We observed significant de- crease in lysosomal acid phosphatase activity in 6-OHDA- and Rotenone-treated cells in all the cell lines. U-87 MG glial cells showed decreased lysosomal acid

phosphatase activity in the PD stress conditions as compared to SK-N-SH and SH- SY5Y dopaminergic neuronal cells (Fig. 3b).

TFEB is a transcription factor and a master regulator of lysosome biogenesis and autophagy, and nuclear translocation of TFEB is essential for activating transcriptional programmes related to lysosomal biogenesis [32]; therefore, we assessed the nuclear localization of TFEB in PD stress conditions using western blotting. SH-SY5Y dopaminergic neuronal cells and U-87 MG glial cells were treated with Rotenone and 6- OHDA, and nuclear fractions were prepared, and the levels of TFEB were monitored using TFEB-specific antibody. Western blot analysis of the nuclear fractions showed de- creased nuclear localization of TFEB in 6-OHDA conditions in both SH-SY5Y and U-87 MG cells (Fig. 3c (i)(a) and (ii)(a)), while no change could be observed in Rotenone- treated conditions. Relative quantification of the western blots confirm that the TFEB/Lamin ratio decreases in 6-OHDA treatment as compared to untreated, and no significant change is observed under Rotenone conditions (Fig. 3c (i)(b) and (ii)(b)).

Altered Autophagic Flux Modulates Exosome Release in PD Stress Conditions

Autophagy is a degradative mechanism used by all cell types to maintain protein homeostasis in the cells. Deficiency of basal autophagy results in neurodegeneration characterized by the ac- cumulation of ubiquitinated-protein aggregates [33]. Above re- sults strongly suggest the lysosomal dysfunction in PD stress conditions, which can lead to autophagy defect; hence, autopha- gy was monitored in neuronal and glial cells in PD stress condi- tions. We observed that 6-OHDA modulates the autophagy flux using tandem-mcherry-GFP-LC3 construct. The yellow puncta (red and green merge) indicate autophagosomes whereas red puncta indicate autophagosomes fused with lysosomes also called as autophagolysosomes [34]. All the cell lines were transfected with mCherry-GFP-LC3 and subsequently treated with 6-OHDA and Rotenone, and red/yellow puncta were mon- itored. In all the three cell lines, the number of red puncta de- creased in 6-

OHDA as well as Rotenone-treated cells as com- pared to untreated. Similarly, the yellow-coloured puncta in- creased per neurons in the 6-OHDA and Rotenone condition, indicating the reduced autophagic flux (Fig. 4a). We also exam- ined autophagy using LC3 western blotting in cell lines treated with 6-OHDA and Rotenone. LC3, the cytosolic form of which (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), is recruited to autophagosomal membranes [35]; hence, LC3 is an established marker of autophagy. In SH-SY5Y dopaminergic neuronal cells, enhanced conversion of LC3-I to LC3-II is observed in 6- OHDA-treated cells, and further accumulation is observed when the autophagy pathway is blocked by Bafilomycin (100 nM) (Fig. 4b (i)(a)) which is also evident by the LC3II/LC3I ratio (Fig. 4b (i)(b)). Similar results are obtained in SK-N-SH dopa- minergic neuronal cells (Fig. 4b (ii)). However, a decrease in LC3-II levels are observed in U-87 MG glial cells, but LC3 accumulates when co-treated with Bafilomycin A1 (Fig. 4b (iii)(a) and (iii)(b)). These results indicate alteration of autophagic flux in all the cell lines under PD stress conditions. The autophagic flux was also monitored using wortmannin, which is a widely used autophagy inhibitor. LC3-II accumulates in cells co-treated with 6-OHDA and wortmannin which indicates defect in autophagic flux in SH-SY5Y dopaminergic neuronal cells (Supplementary Fig. S2 (a)(i) and (a)(ii)). There are emerging evidences to show a molecular and functional crosstalk between autophagy pathways and exosome release [36]. Exosome release may serve as a cellular mechanism to partially bypass the autoph- agic defect that occurs during pathological situations. To eluci- date the crosstalk of the autophagy and exosome release path- ways under PD stress conditions, SH-SY5Y and SK-N-SH do- paminergic neuronal cells and U-87 MG glial cells were treated with 6-OHDA and Rotenone, and autophagy pathway was blocked using Bafilomycin, and subsequent exosome release was monitored by western blotting. In all the cell lines, enhanced exosome release was observed when autophagy pathway is blocked by Bafilomycin and even more enhanced when the PD stress conditions are co-treated with Bafilomycin (Fig. 4c (i)(a), (ii)(a) and (iii)(a)). Relative quantification of the CD63 levels as compared to control confirms that there is a significant increase in the co-treated conditions (Fig. 4c (i)(b), (ii)(b) and (iii)(b)). Similarly, subsequent exosome release was checked in all the cell lines under PD stress conditions co-

treated with wortmannin. The cells treated with wortmannin did not significantly alter the exosome release in the cells whereas decreased in cells co-treated with 6-OHDA and Rotenone with wortmannin (Supplementary Fig. S2 (d), (e), and (f)).

Enhancement of Autophagy Flux by Rapamycin Decreases the Release of Exosomes in PD Stress Conditions

The experiments here showed that mitochondrial and lysosomal dysfunction can modulate the autophagic flux and hence also modulate the exosomal release in PD stress conditions. We hy- pothesized that enhancing the basal autophagic flux may de- crease the exosomal release. Rapamycin is an allosteric inhibitor of mammalian target of rapamycin (mTOR), and inhibition of mTOR activity enhances autophagy, which in turn plays a role in maintaining metabolic homeostasis [37]; however, its implica- tion in exosomal release in neuronal cells is not well understood. Hence, we treated the cells with PD stress conditions in presence and absence of rapamycin and checked the exosome release. Firstly, we analysed if rapamycin can modulate the autoph- agy flux in both neurons and glial cells. SH-SY5Y dopami- nergic neuronal cells treated with 6-OHDA showed en- hanced level of LC3-II form which decreased when cells were co-treated with rapamycin (Fig. 5a (i)). Similarly, en- hanced degradation of accumulated LC3-II forms was ob- served in U-87 MG glial cells (Fig. 5a (ii)). These evidences suggest that rapamycin enhances the autophagy flux both in neuronal and glial cells in the presence of PD stress condi- tions. As enhanced autophagy flux may modulate the re- lease of exosomes, hence, we analysed the exosomal re- lease in presence of PD stress conditions and rapamycin. In SH-SY5Y dopaminergic neuronal cells and U-87 MG glial cells, the level of CD63 marking the exosome release is enhanced in the presence of 6-OHDA and Rotenone and PD stress conditions. Interestingly, the level of CD63 is decreased in cells co-treated with 6-OHDA and Rotenone with rapamycin both in SH-SY5Y and U-87 MG cells (Fig. 5b (i) and (ii)). These experiments suggest that enhancing the autophagy through rapamycin decreased the release of exosome both in

neuronal and glial cells.

Discussion

Parkinson's disease is typically characterized by the death of dopaminergic neurons in the substantia nigra part of the brain; however, it has been well established that visual impairments occur commonly in PD, along with loss of DA-producing retinal amacrine cells in the inner nuclear and ganglion cell layers and secondary depletion of the dopaminergic fiber plexus of the inner plexiform layer. Similarly, neuronal loss and Lewy body pathol- ogy has been observed in the anterior olfactory nucleus as well as the olfactory bulb [38]. The spread of the pathology to different cell types suggest the importance of inter-neuronal communica- tion through exosomes. The exosomal release is a complex crosstalk of mitochondria, lysosome and MVBs to counter the cellular stress and cell may release the damaged organelle/ proteins along with other cargo to protect the cells. In the current study, we studied the mitochondria-lysosomal crosstalk in the release of exosome in PD stress conditions.

We used neuronal and glial cell lines for the study since it is now well established that glial cells play a role in PD. Glial reaction occurs as a result of the neuronal cell death in neuro- degenerative diseases, and it has been found that even after the initial insult to the neuron has disappeared, the glial reaction initiated further propagates the neuronal degeneration [39, 40]. Moreover, it is now known that the density of microglial cells is remarkably higher in the substantia nigra as compared to the other regions of the brain-like hippocampus and the midbrain regions, which also suggest that the substantia nigra neurons are more susceptible to microglial-mediated injuries, supporting the implication of gliosis in PD pathogenesis [41]. We first isolated and characterized the exosomes released from both cell types. Isolation of exosomes was performed by an affinity purification-based method. The exosomes were subsequently characterized by Nanoparticle Tracking Analysis and western blotting against the well-characterized exosomal marker, CD63. We also checked the exosome re- lease in the presence of 6-OHDA and Rotenone, which are the two

chemicals used to mimic the Parkinson's disease condi- tions. All the evidences here strongly suggest the enhanced exosomal release in PD stress conditions in dopaminergic neuronal cells (SH-SY5Y and SK-N-SH) and glial cells

(U-87 MG).

Previous reports have shown that mitochondria in the neu- rons are more susceptible to oxidative stress as compared to other cell types [42]. Fragmented mitochondria are usually followed by a decrease in respiration and eventual cell death and is observed in PD models [43]. In our study, we show that mitochondrial fragmentation occurs in the U-87 MG glial cells on treatment with 6-OHDA and Rotenone. Furthermore, con- sistent with previous reports [44–46], we showed that SH- SY5Y and SK-N-SH dopaminergic neuronal cells treated with 6-OHDA and Rotenone show impaired mitochondrial com- plex I activity and ATP level decline and oxidative stress which is the hallmark in PD pathogenesis [47, 48]. An asso- ciation between PD and hindered complex I activity in the brain was reported by several groups [49, 50]. Chemicals and environmental toxins including Rotenone, 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat and nitric oxide are all shown to inhibit mitochondrial functions which show Parkinson-type symptoms [51]. This impaired mitochondrial respiration leads to reduction of ATP levels [46] and is one of the major causes of neurodegeneration, including PD [52].

Mitochondrial OXPHOS activity is known to maintain NADH/NAD ratio which is also important for the main- tenance of the lysosomal functions [30]. The crosstalk is further established by recent report where lysosomal dys- function can lead to generation of a LIPL-4, a lipid sig- nalling messenger which adjust the mitochondrial ETC activity and mitochondrial ß-oxidation to reduce lipid storage and promote longevity in *Caenorhabditis elegans* [53]. Therefore, the evidence here and emerging reports strongly suggest the mitochondria and lysosomal crosstalk in regulation of neuronal homeostasis; however, its impli- cation of this signalling in PD pathogenesis needs to be further understood.

Lysosome is now becoming one of the important organ- elles in cellular homeostasis. The dysfunction in lysosomes may lead to alteration of several pathways leading to cell death and progression of several chronic

diseases. The dysfunction leads to decrease in autophagy flux and hence accumulation of autophagosomes. We showed the dysfunction in lysosomes in neuronal as well as glial cell lines by using LysoTracker Blue dye and counting average number of lysosomes per cell through confocal microscopy. Acid phosphatase activity was also hampered in all the three cell lines in PD stress condi- tions. TFEB is a major regulator in coordinating autophagy induction along with lysosomal biogenesis, and its activation has ameliorated various neurodegenerative disorders in mouse models [54]. By nuclear fractionation of the cells and western blot analysis, we showed that the nuclear translocation of TFEB is perturbed in 6-OHDA conditions, indicating defec- tive lysosomal biogenesis and autophagy flux. This was fur- ther confirmed by monitoring the autophagy flux in all three cell lines in 6-OHDA and Rotenone conditions using the tan- dem construct mCherry-GFP-LC3. The number of yellow puncta considerably increased in 6-OHDA and Rotenone con- ditions indicating the reduced autophagic flux and impaired autophagy.

How the impairment of autophagy in PD stress conditions affects the exosome release is not well understood. Hence, we assessed the exosome release under the same conditions. The blocking of autophagy pathway by Bafilomycin A1 greatly enhances the exosome release in the dopaminergic neuronal and glial cells.

The class III PI3Ks are known to play a role in recruitment of specific effector proteins to promote endocytosis, endo- some fusion, and maturation, as well as cargo sorting to lyso- somes, and moreover are essential for the ILV formation [55]. We observed here that inhibition of class III PI3Ks with the help of wortmannin resulted in decreased release of exosomes in PD stress conditions. This indicates that the regulation of exosome release is largely dependent on the inhibition of the autophagy process at the terminal step of fusion of the autophagosome with the lysosome. The results here suggest that wortmannin, which blocks autophagy at the initiation stage, also may affect a number of other different pathways because of their broad-spectrum action, and hence the en- hanced exosome release is not observed in these cases.

The enhancement of autophagy by rapamycin in PD stress conditions in both SH-SY5Y dopaminergic neuronal cells and U-87 MG glial cells showed a decrease in the release of exosomes. This is in

consonance with previous reports where it has been observed that rapamycin plays a protective role against cell death in in vitro and in vivo models of PD [37], and this may be because of preventing the exosomal release by enhancing the autophagy flux. This suggests that enhance- ment of autophagy pathway by Rapamycin could lower the exosome release content by the cells in PD stress conditions, which could provide a therapeutic possibility to stop the spread of the pathogenesis of the disease through exosomes.

Conclusion

In conclusion, the study here demonstrates that inter- organellar crosstalk between mitochondria, lysosomes and MVB is now emerging important in PD pathogenesis. The mutation in several proteins regulating mitochondrial dys- function may in turn inhibit the lysosomal function leading to decreased autophagy flux; hence, increased exosomal re- lease is observed, containing several pathogenic cargo com- ponents which may intensify the pathogenesis of PD. The evidences here suggest that the understanding of the inter- organellar crosstalk may provide unique opportunity to mod- ulate the combinatorial strategy to enhance the autophagy flux and prevent exosomal release hereby reducing the spread of PD and helping ameliorate the disease.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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Figure legends:

Fig. 1 Exosome release is enhanced in Parkinson's disease stress,, conditions: a Schematic flowchart of the exosome isolation. b Characterization of exosomes by (i) Nanoparticle Tracking Analysis— U-87 MG exosomes were suspended in DPBS and analysed by NTA. The exosome size was found to be in a range of 80–150 nm. (ii) Western Blotting—Exosomes were isolated from SK-N-SH cells, and western blotting was performed and blotted against antibodies as indicated. c Exosomes were isolated from (i) SH-SY5Y, (ii) SK-N-SH and (iii) U- 87 MG cells treated with 6-OHDA and Rotenone, and exosome concen- tration was monitored by acetylcholine esterase activity. Asterisk (*) and (**) indicates units statistically significant from control; p value < 0.05 and < 0.01 (respectively), SEM of two independent experiments. d Exosomes were isolated from (i) SH-SY5Y, (ii) SK-N-SH and (iii) U- 87 MG cells treated with 6-OHDA and Rotenone and subjected to NTA analysis. Asterisk (*) and (***) indicates levels statistically significant from control; p value < 0.05 and < 0.001 (respectively), SEM of three independent experiments. e Exosomes were isolated from (i)(a) SH- SY5Y, (ii)(a) SK-N-SH and (iii)(a) U-87 MG cells treated with 6- OHDA and Rotenone and were subjected to western blot analysis using the indicated antibodies. Relative quantification of CD63 as compared to untreated samples in (i)(b) SH-SY5Y, (ii)(b) SK-N-SH and (iii)(b) U-87 MG exosome lysates of cells treated with 6-OHDA and Rotenone. Asterisk (*), (**) and (***) indicates units statistically significant from control; p value < 0.05, < 0.01 and < 0.001 (respectively), SEM of three independent experiments

Fig. 2 Parkinson's disease stress conditions lead to mitochondrial dysfunctions: a MT-RFP transfected U-87 MG cells were treated with 6-OHDA and Rotenone and analysed by confocal microscopy. b (i) SH-SY5Y and (ii) SK-N-SH cells were treated with 6-OHDA and Rotenone, and complex I activity was measured by spectrophotometric method. Asterisk (*) indicates levels statistically significant from control; p value < 0.05, SEM of three independent experiments. c (i) SH-SY5Y, (ii) SK- N-SH and (iii) U-87 MG cells were treated with 6-OHDA and Rotenone, and ATP levels were determined by

luminescence method. Asterisk (**) and (***) indicates levels statistically significant from control; p value < 0.01 and < 0.001 (respectively), SEM of three independent experiments

Fig. 3 Lysosomal functions are altered in Parkinson's disease stress conditions: a (i) SH-SY5Y, (ii) SK-N-SH and (iii) U-87 MG cells were treated with 6-OHDA and Rotenone for 24 h. After treatment cells were stained with LysoTracker Blue as described in method section and were visualized by confocal microscopy and average number of lysosomes per cell was quantified using ImageJ software. Asterisk (***) indicates num- ber of puncta statistically significant from control; p value < 0.001, SEM of three independent experiments. b (i) SH-SY5Y, (ii) SK-N-SH and (iii) U-87 MG cells were treated with 6-OHDA and Rotenone for 24 h. After treatment, acid phosphatase activity was determined as described in the "Materials and methods" section. Asterisk (*) and (***) indicates acid phosphatase levels statistically significant from control; p value < 0.05 and < 0.001 (respectively), SEM of four independent experiments. c (i)(a) SH-SY5Y and (ii)(a) U-87 MG cells were treated with 6-OHDA and Rotenone, and nuclear fractionation was performed and subjected to western blot analysis using the indicated antibodies. Relative quantifica- tion of TFEB protein levels in (i)(b) SH-SY5Y and (ii)(b) U-87 MG cells treated with 6-OHDA and Rotenone. Asterisk (*) and (**) indicates units statistically significant from control; p value < 0.05 and < 0.01 (respec- tively), SEM of two independent experiments

Fig. 4 Altered autophagic flux modulates exosome release in PD stress conditions: a Quantification of red and green puncta in mCherry-GFP-
LC3 transfected cells in the presence and absence of 6-OHDA and Rotenone. The numbers of LC3 puncta per cell were counted, and graph was plotted for numbers of mCherry-LC3 puncta per cell in (i) SH-SY5Y,

(ii) SK-N-SH and (iii) U-87 MG. Asterisk (*), (**) and (***) indicates number of LC3 puncta statistically significant from control; p value < 0.05, < 0.01 and < 0.001 (respectively), SEM of three independent exper- iments. b (i)(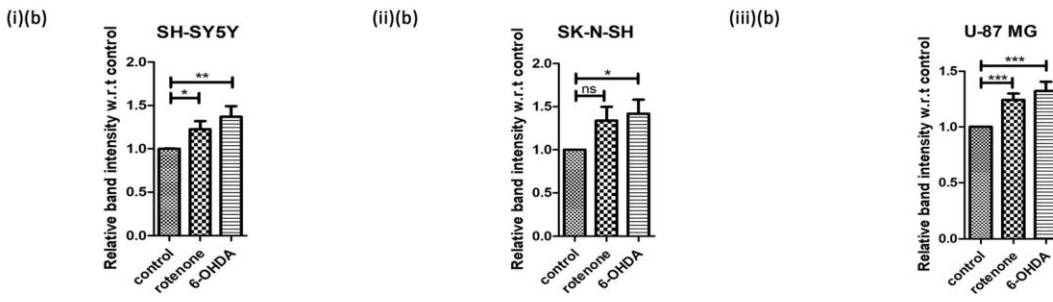a) SH-SY5Y, (ii)(a) SK-N-SH and (iii)(a) U-87 MG cells were treated with Rotenone and 6-OHDA and western blot analysis of autophagy marker, LC3 was performed in presence and absence of Bafilomycin A1. Relative quantification of LC3 II/ LC3 I ratio in (i)(b) SH-SY5Y, (ii)(b) SK-N-SH and (iii)(b) U-87 MG cells treated with 6- OHDA and Rotenone in the presence and absence of Bafilomycin A1. Asterisk (*) and (**) indicates levels statistically significant from control; p value < 0.05 and < 0.01 (respectively), SEM of three independent ex- periments. c (i)(a) SH-SY5Y, (ii)(a) SK-N-SH and (iii)(a) U-87 MG cells were treated with Rotenone and 6-OHDA, and subsequent exosome re- lease was monitored in the presence and absence of Bafilomycin A1 by performing western blot against the exosome marker, CD63. Relative quantification of CD63 as compared to untreated samples in (i)(b) SH- SY5Y, (ii)(b) SK-N-SH and (iii)(b) U-87 MG exosome lysates of cells treated with 6-OHDA and Rotenone in the presence and absence of Bafilomycin A1. Asterisk (*), (**) and (***) indicates levels statistically significant from control; p value < 0.05, < 0.01 and < 0.001 (respective- ly), SEM of three independent experiments

Fig. 5 Enhancement of autophagy flux by rapamycin decreases the release of exosomes in PD stress conditions: a (i)(a) SH-SY5Y and (ii)(a) U-87 MG cells were treated with Rotenone and 6-OHDA, and western blot analysis of autophagy markers was performed in the pres- ence and absence of rapamycin. Relative quantification of LC3 II/ LC3 I ratio in (i)(b) SH-SY5Y and (ii)(b) U-87 MG cells treated with 6-OHDA and Rotenone in the presence and absence of rapamycin, SEM of three independent experiments. b (i)(a) SH-SY5Y and (ii)(a) U-87 MG cells were treated with Rotenone and 6-OHDA, and subsequent exosome re- lease was monitored in the presence and absence of rapamycin by performing western blot against the exosome marker, CD63. Relative quantification of CD63 as compared to untreated samples in (i)(b) SH- SY5Y and (ii)(b) U-87 MG exosome lysates of cells treated with 6-

OHDA and Rotenone in the presence and absence of rapamycin. Asterisk (*), (**) and (***) indicates levels statistically significant from control; p value < 0.05, < 0.01 and < 0.001 (respectively), SEM of three independent experiments

Fig.1

Fig.2

(a)

CONTROL | ROTENONE | 6-OHDA

MT-RFP

10 µm | 10 µm | 10 µm

(b) (i) SH-SY5Y

(ii) SK-N-SH

(c) (i) SH-SY5Y

(ii) U-87 MG

(iii) SK-N-SH

Fig.3

(a) (i) UNTREATED 6-OHDA ROTENONE — LysoTracker Blue

SH-SY5Y — Average number of lysosomes per cell — control, 6-OHDA, Rotenone — ***

(ii) UNTREATED 6-OHDA ROTENONE — LysoTracker Blue

SK-N-SH — Average number of lysosomes per cell — control, 6-OHDA, Rotenone — *** ns

(iii) UNTREATED 6-OHDA ROTENONE — LysoTracker Blue

U-87 MG — Average number of lysosomes per cell — control, 6-OHDA, Rotenone — *** ***

(b) (i) SH-SY5Y — Relative acid phosphatase levels — control, Rotenone, 6-OHDA — *** *

(ii) SK-N-SH — Relative acid phosphatase levels — control, rotenone, 6-OHDA — *** ns

(iii) U-87 MG — Relative acid phosphatase levels — control, rotenone, 6-OHDA — *** ***

(c) (i)(a) control Rotenone 6-OHDA control Rotenone 6-OHDA
α- TFEB — 53 KDa
α- Lamin — 70 KDa
α- Actin — 42 KDa
cytosol nucleus
SH-SY5Y

(ii)(a) control Rotenone 6-OHDA control Rotenone 6-OHDA
α- TFEB — 53 KDa
α- Lamin — 70 KDa
α- Actin — 42 KDa
cytosol nucleus
U-87 MG

(i)(b) SH-SY5Y — TFEB/Lamin — control, rotenone, 6-OHDA — ns *

(ii)(b) U-87 MG — TFEB/Lamin — control, rotenone, 6-OHDA — ns **

Fig.4

Fig.5

ions.