Chaperone-Mediated Autophagy Markers in Parkinson Disease Brains

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Objective: To investigate chaperone-mediated autophagy in the pathogenesis of Parkinson disease (PD).

Design: Postmortem observational study.

Setting: University Department of Clinical Neuroscience, Institute of Neurology, University College London.

Subjects: Postmortem samples from 7 PD, 6 Alzheimer disease (AD), and 8 control brains.

Main Outcome Measure: Lysosomal-associated membrane protein 2A (LAMP2A) and heat shock cognate 70 (hsc70) protein levels were compared in the substantia nigra pars compacta and amygdala of PD, AD, and control brain samples. To provide insight into the turnover of α-synuclein, degradation pathways for this protein were studied in a dopaminergic cell line.

Results: The expression levels of the chaperone-mediated autophagy proteins LAMP2A and hsc70 were significantly reduced in the substantia nigra pars compacta and amygdala of PD brains compared with age-matched AD and control brain samples. Lewy bodies in these regions contained autophagy-related proteins. We demonstrated that decreased LAMP2A levels in dopaminergic cell lines reduced chaperone-mediated autophagy activity and increased the half-life of α-synuclein.

Conclusions: These findings suggest that there is reduced chaperone-mediated autophagy activity in the PD brain, provide evidence for the role of autophagy in PD pathogenesis and Lewy body formation, and suggest that this pathway may be a suitable therapeutic target in PD.


The identification of α-synuclein gene mutations and multiplications as a cause of familial Parkinson disease (PD) and the presence of this protein as a major constituent of Lewy bodies (LBs) in the PD brain have focused attention on the intracellular metabolism of this protein as a central component of the pathogenetic pathway(s) of PD. Intracellular protein degradation is achieved through a combination of the ubiquitin-dependent proteasomal system (UPS) and autophagic pathways. Abnormalities of UPS function have been reported in the PD brain,1 and inhibitors of the UPS have been reported to induce a rodent model of PD in some2,3 but not all studies.4,5 However, the role of the UPS in α-synuclein metabolism remains unclear. Attention has recently focused on the potential contribution of autophagy to the pathogenesis of neurodegenerative diseases.6-8 Autophagy is composed of 3 separate pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy involves the formation of double-membraned autophagosomes, which fuse with lysosomes to deliver cytoplasmic contents, including misfolded or aggregated proteins for digestion and recycling of amino acids. Microautophagy involves lysosomal pinocytosis of cytoplasmic contents and is involved in the turnover of long half-life cytosolic proteins. Chaperone-mediated autophagy is dependent on the heat shock cognate 70 (hsc70) protein and its binding to lysosomal-associated membrane protein 2A (LAMP2A), a lysosomal surface receptor. A highly specific subset of cytosolic proteins with a KFREQ motif are recognized by the hsc70 chaperone and internalized for degradation by LAMP2A lysosomal membrane receptors.9

α-Synuclein has a pentapeptide (95VKKDQ99) sequence consistent with hsc70 binding.10 Using isolated lysosomal preparations, wild-type (WT) α-synuclein has been shown to be degraded following binding to the chaperone-mediated autophagy LAMP2A receptor11; this result has been confirmed in neuronal cells.12 However, mutant α-synuclein bound to this lysosomal receptor with high affinity but was not translocated across the membrane and appeared to block these receptors, thereby inhibiting the chaperone-mediated autophagy pathway.13 This has...
led to the suggestion that decreased chaperone-mediated autophagy may prevent the clearance of α-synuclein and contribute to oligomer and aggresome formation and the pathogenesis of PD. The increase in autophagic vacuoles in the substantia nigra of PD brains and toxin and transgenic models of PD supports the notion that autophagy pathways are important in PD. We hypothesized that chaperone-mediated autophagy activity may be reduced in PD brains and contribute to both LB formation and the pathogenesis of neuronal degeneration.

METHODS

PATIENT BRAIN SAMPLES

Substantia nigra pars compacta and amygdala samples from normal controls and patients with PD or Alzheimer disease (AD) who had died were obtained from the Brain bank University Hospital of Bellvitge, Barcelona, Spain, and the Navarra Brain Bank, Pamplona, Spain, with the consent of the local ethics committees. Controls had no clinical evidence ante mortem or pathological evidence post mortem of any neurodegenerative disease. Pathological diagnoses of PD and AD were made according to recognized criteria (Queen Square Brain Bank criteria).

CELL CULTURES

SH-SY5Y clones constitutively expressing either full-length human WT or A53T mutant α-synuclein with a C-terminal hemagglutinin tag were grown under standard conditions in the presence of G418. Other reagents were obtained from Sigma Aldrich (Dorset, England) or Merck (Nottingham, England) unless otherwise stated.

SAMPLE PREPARATION

Brain or cell samples were solubilized in a 10mM Tris/HCl (pH 7.4) buffer containing 0.1% sodium dodecyl sulfate, a protease inhibitor mixture, and DNase (Promega, Epsom, England) or negative control siRNA No. 1 (Ambion). Optimal concentrations of siRNA were identified between 5nM and 100nM siRNA during 72 hours. Total messenger RNA was isolated using the RNeasy Mini Kit (Qiagen), complementary DNA was obtained using SuperScript III Reverse Transcriptase (Invitrogen), and LAMP2A messenger RNA levels quantified by real-time polymerase chain reaction (QuantiTect SYBR Green PCR Kit, Qiagen) using a forward primer for exon 8 (TGCCTTGGCAGGAGTACTTA) and a reverse primer for exon 9 (TCTCAAAATGCTGGGATGTAT), relative to GAPDH messenger RNA levels. For long-term LAMP2A depletion, cells were transfected with 20nM siRNA at 0, 3, and 6 days.

CELL PROLIFERATION ANALYSIS

After 6 days of siRNA treatment, cells were seeded in a 96-well plate and treated with siRNAs until day 9. Proliferation rates were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay17 and expressed as a percentage of the control for each cell line.

STUDY OF α-SYNUCLEIN AND GAPDH HALF-LIFE

α-Synuclein and GAPDH turnover was assessed using a standard cycloheximide (25 µg/ml) procedure.18 Samples were removed at 12-hour intervals up to 48 hours. Sample loadings from each cell number were calculated using the fluorescent DAPI assay of DNA content using a Synergy plate reader (Biotek, Potton, England). Western blots were analyzed using DigiDoc gel analysis software, and half-life was calculated using linear regression model; average half-life was calculated from regression lines drawn for each individual experiments.20 All experiments were performed in triplicate. The involvement of various degradation pathways was studied in the presence of the following inhibitors: 10mM benzylxoycarbonyl-I-isoleucyl-γ-t-butyI-L-glutamyl-L-alanyl-levulinic acid (PSI); 10mM 3-methyl-adene, and 20mM ammonium chloride or siRNA to LAMP2A.

WESTERN BLOT ANALYSIS

Samples were separated on NuPAGE Novex 4%-12% Bis-Tris Gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Millipore, Watford, England). Primary antibodies were antimagglutinin (1:5000; Covance, Princeton, New Jersey); anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10000); anti-LAMP2A (1:400); anti-hsc70 (1:500); anti–β-actin (1:2000) (all Abcam, Cambridge, England); anti-lysosomal-associated membrane protein 1 (LAMP1) (1:250; BD Biosciences, Oxford, England); and microtubule-associated light chain LC3 (1:500; Cell Signaling, Danvers, Massachusetts). Membranes were incubated with horseradish peroxidase–conjugated antirabbit or antirabbit antibodies (DAKO, Ely, England), which were detected using ECL Plus Western Blot Detection Reagents and Hyperfilm ECL (GE Healthcare, Little Chalfont, England). Films were scanned and signals in the linear range were quantified using DigiDoc gel analysis software (Alpha Innotech, San Leandro, California). Data were normalized to β-actin levels. Proteins levels were determined by the Bradford assay (Biorad, Hemel Hempstead, England) using bovine serum albumen as the standard.

INHIBITION OF LAMP2A EXPRESSION BY siRNAs

Cells were transfected using HiPerfect Transfection reagent (Qiagen, Hiklen, Germany) with 2 small interfering RNAs (siRNAs) against exon 9a of the LAMP2A gene (GCACCAUCAUGCG-GAUUAATdTdT and CUAAGAGGGCCUCGGCAAAdTdT; Dharmacon, Epsom, England) or negative control siRNA No. 1 (Ambion).
secondary antibodies (diluted 1:400; Alexa488 antirabbit and Alexa546 antitmuse; Invitrogen, Barcelona). Nuclei were stained with TO-PRO-3 iodide (diluted 1:1000; Invitrogen). After washing in phosphate-buffered saline, the sections were mounted in Immuno-Fluore Mounting medium (ICN Biomedicals Inc), sealed, and dried overnight before analysis with a Leica TCS-SL confocal microscope. For immunocytocchemistry, SH-SY5Y cells were seeded on coverslips and immunostained using the anti–α-synuclein antibody (1:200; Zymed, Paisley, Scotland) as previously described.16

STATISTICAL ANALYSIS

Statistical analyses of the data were performed using SPSS, program 16.0, using the nonparametric Kruskal Wallis test followed by the Mann-Whitney U test.

LEVELS OF CHAPERONE-MEDIATED AUTOPHAGY PROTEINS IN THE PD BRAIN

To identify whether the chaperone-mediated autophagy pathway was abnormal in the PD brain, we determined the levels of 2 key proteins, LAMP2A and hsc70, in the substantia nigra pars compacta and amygdala of PD, AD, and control brain samples. These regions were selected because the substantia nigra pars compacta is the site of major dopaminergic cell death and LB deposition in PD, while the amygdala accumulates LBs but does not undergo significant neurodegeneration. Samples from AD brains were used as a disease control. In these brains, the amygdala showed typical AD pathology with neuronal loss, neurofibrillary tangles, and amyloid plaques without any LBs. The substantia nigra pars compacta in AD brains showed mild neuronal loss, variable numbers of globose tangles, and phosphorylated tau–immunoreactive threads, but LBs and Lewy neurites were absent. The 3 groups were matched for postmortem delay (mean [SD]: control, 4.9 [1.8] hours; PD, 4.35 [3.1] hours; and AD, 2.95 [0.7] hours). The PD and control brains were matched for age (mean [SD]: control, 70.5 [8.7] years; PD, 77.4 [3.7] years), but the AD brains were older (87.2 [5.3] years; P<.05), a reflection of the disease characteristics and age at death. Four of the PD brains were at Braak stage 4, 1 was at stage 5, and 1 was at stage 6. The 6 AD brains were all at Braak stage VI B and C.

Both LAMP2A and hsc70 levels were significantly decreased in PD substantia nigra pars compacta and amygdala compared with control samples (Figure 1A and B). Relative to controls, no significant changes were seen in the AD substantia nigra pars compacta or amygdala with respect to LAMP2A or hsc70 levels. To determine whether the reduction of LAMP2A in PD reflected decreased lysosomal levels of this protein, pellets enriched with the lysosomal fraction were isolated from the amygdala of control, AD, and PD samples and probed for LAMP2A and LAMP1 (see the “Methods” section). This confirmed that the lysosomal fraction of LAMP2A levels was decreased in PD but not AD (Figure 1C).

MARKERS OF AUTOPHAGY IN PD BRAIN

LC3-II is a component of the autophagosome membrane and has been characterized as a marker of macroautophagy in mammals.21 LC3-II levels were elevated in the substantia nigra pars compacta and amygdala of PD brain samples, suggesting an increase in macroautophagy, but they did not reach statistical significance (Figure 1D). There was no significant change in the AD samples.

Immunofluorescence and confocal microscopy demonstrated that LC3 colocalized with α-synuclein in most LBs and Lewy neurites in PD substantia nigra pars compacta as well as in small punctate α-synuclein immunoreactive inclusions (Figure 2A-C); LAMP2A was absent from most LBs but it colocalized with α-synuclein in some abnormal inclusions (Figure 2D-F); and hsc70 immunoreactivity occurred in a minority of α-synuclein inclusions, but it was largely absent in LBs (Figure 2G-I).

CHAPERONE-MEDIATED AUTOPHAGY AND α-SYNUCLEIN TURNOVER IN DOPAMINERGIC CELLS

To determine the potential impact of reduced LAMP2A levels on α-synuclein turnover, we established a cell model with decreased chaperone-mediated autophagy by knockdown of LAMP2A protein using siRNAs. In human dopaminergic neuroblastoma SH-SY5Y cells, exposure to these siRNAs for 72 hours decreased LAMP2A messenger RNA levels by 80%. (eFigure, A, available at http://www.archneurol.com). This level of silencing was maintained when the cells were treated for 9 days with siRNA applications every 3 days (eFigure, B). Chaperone-mediated autophagy activity was assessed indirectly by measuring the turnover of a well-characterized chaperone-mediated autophagy substrate, GAPDH.21 In WT α-synuclein–overexpressing SH-SY5Y cells, siRNA silencing resulted in up to a 95% decrease in LAMP2A messenger RNA level (eFigure, C), a LAMP2A protein level that was undetectable (eFigure, D), and a significant increase in mean GAPDH half-life, from 48.1 hours (SD, 10.9 hours) to 116 hours (SD, 16.2 hours) (eFigure, E). Cell proliferation between 6 and 9 days of LAMP2A siRNA treatment was not affected in control SHSY5Y cells, but was reduced in WT α-synuclein–expressing cells (eFigure, F). This was not associated with any visible evidence of increased cell death (data not shown).

To investigate the degradation pathways of WT α-synuclein in intact cells, we analyzed its half-life in the presence of inhibitors of the proteosome (with PSI), lysosomal function (with ammonium chloride), macroautophagy (with 3-methyladenine), and chaperone-mediated autophagy (with LAMP2A siRNAs). Under normal conditions, the mean half-life of WT α-synuclein was 46.5 hours (SD, 2.4 hours), which was not significantly influenced by inhibition of the UPS or macroautophagy, but was almost doubled (mean [SD], 80 [6.6] hours, P<.05) by ammonium chloride (Figure 3A). Downregulation of LAMP2A increased mean α-synuclein half-life to 65 hours (SD, 12.1 hours)
Following LAMP2A depletion the half-life of WT α-synuclein was not further increased with inhibition of the UPS. However, inhibition of macroautophagy partially increased α-synuclein half-life, suggesting that WT α-synuclein turnover was more dependent on macroautophagy when chaperone-mediated autophagy was inhibited (Figure 3A and B). As expected, following ammonium chloride treatment, the decrease in LAMP2A had no further impact on α-synuclein half-life. The decreased turnover of α-synuclein was associated with a concomitant increase in α-synuclein levels with increased cytoplasmic and perinuclear α-synuclein accumulation on immunohistochemistry (Figure 3C and D).

These results confirm in human-derived dopaminergic cells that WT α-synuclein is predominantly degraded by chaperone-mediated autophagy and not by the proteasome or macroautophagy. In contrast, A53T α-synuclein is degraded by macroautophagy and the proteasome, but not by chaperone-mediated autophagy (Figure 1).

Figure 1. Chaperone-mediated autophagy markers in Parkinson disease (PD) and Alzheimer disease (AD) brain. A, Analysis of chaperone-mediated autophagy–related proteins (lysosomal-associated membrane protein 2A [LAMP2A] and heat shock cognate 70 [hsc70]) in the substantia nigra pars compacta in control (n=8), AD (n=6), and PD (n=7) samples, expressed relative to actin levels. B, Levels of LAMP2A and hsc70 relative to actin in the amygdala of control (n=7), AD (n=6), and PD (n=6) samples. C, The level of LAMP2A relative to lysosomal-associated membrane protein 1 (LAMP1) in the amygdala lysosomal (LSS) fraction isolated from control (n=7), AD (n=6), and PD (n=6) samples. D, Amount of the conjugated 16-kDa form of the microtubule-associated protein 1 light chain 3 (LC3-II) relative to actin in the substantia nigra pars compacta and amygdala. AU, arbitrary units; WB signal, band intensity of Western blots. *P<.05, vs controls; †P<.01, vs controls; error bars, SD.
Figure 2. Autophagy markers in the substantia nigra pars compacta in Parkinson disease (PD) brain samples. Sections of the substantia nigra pars compacta were costained with LC3 (A, green) and α-synuclein (B, red) (merged confocal images [C]); and lysosomal-associated membrane protein 2 (LAMP2) (D, green) and α-synuclein (E, red) (merged confocal images [F]). The arrow indicates that LAMP2 was present in some α-synuclein-immunoreactive inclusions but not in most Lewy bodies (F). Heat shock cognate 70 (hsc-70) (G, green), α-synuclein (H, red), and colocalization (J). J-L, As a negative control, sections were stained without the primary antibodies. Nuclei were stained with TO-PRO. Scale bars: A-C, 20 µm; D-L, 40 µm.
turnover was not significantly influenced by chaperone-mediated autophagy inhibition by LAMP2A knock-down but was significantly increased in the presence of 3-methyladenine or ammonium chloride (Figure 3E and F). This indicates that A53T/H9251-synuclein was degraded by macroautophagy and not by chaperone-mediated autophagy.

**α-SYNUCLEIN EXPRESSION AND CHAPERONE-MEDIATED AUTOPHAGY ACTIVITY**

We investigated the effect of mild and high expression levels of WT and expression of A53T/H9251-synuclein on chaperone-mediated autophagy in SH-SY5Y cells. Mildly increased expression of WT α-synuclein (clone C111) had no significant impact on GAPDH half-life, but this was slightly increased by high expression of WT (clone C205) and significantly increased by A53T (M114) α-synuclein (Figure 4A-C). These results confirm that the turnover of chaperone-mediated autophagy substrates was decreased by elevated levels of WT α-synuclein and markedly by the A53T mutant protein. The decrease in chaperone-mediated autophagy associated with A53T and high levels of WT α-synuclein expression was associated with mildly elevated LAMP2A or hsc70 levels (Figure 4D) rather than the decrease seen in PD brain samples, suggesting that increased α-synuclein levels alone are not sufficient to decrease the LAMP2A levels seen in PD.

**COMMENT**

The etiology and pathogenesis of PD most likely involve both genetic and environmental factors. Familial PD has been associated with α-synuclein gene mutations and polymorphisms and mutations affecting parkin, LRRK2, and the mitochondrial proteins PINK1, DJ1, and HtrA2. Environmental factors associated with par-
Parkinsonism predominantly affect mitochondrial function, emphasizing this as an important pathway to PD. The role of autophagy and lysosomal function in PD pathogenesis has received support from the relationship between glucocerebrosidase mutations, the cause of the lysosomal storage disorder Gaucher disease, and a significantly increased risk of PD. In addition, mutations of the gene for the lysosomal protein ATP13A2 cause a young-onset parkinsonian disorder with dementia, thought to be mediated by impaired UPS and lysosomal function.

The present study demonstrates that lysosomal LAMP2A and hsc70 protein expression are significantly reduced in PD brains and this is the first direct link between a defect in chaperone-mediated autophagy and PD. This reduction was seen in the substantia nigra pars compacta and amygdala, sites of α-synuclein aggregation and LB deposition. Similar changes were not seen in the same sites in age-matched controls or patients with AD, suggesting some specificity for PD and an association with LB formation. The concomitant trend for increased LC3-II levels suggests a compensatory induction of macroautophagy, though given the presence of LBs, it seems that this did not compensate for the reduction in chaperone-mediated autophagy related to the loss of LAMP2A and hsc70. An induction of LC3-II would be in keeping with the increased numbers of autophagic vacuoles in dopaminergic neurons in PD brain. Upregulation of macroautophagy following chaperone-mediated autophagy inhibition has been seen in fibroblasts and enabled these cells to survive, though they were more susceptible to oxidative stress.

The alteration in chaperone-mediated autophagy protein expression in substantia nigra pars compacta and amygdala in PD indicates that the changes are not specifically related to dopaminergic cell loss and are greater than would be expected with age alone. These studies support the hypothesis that impaired chaperone-mediated autophagy function leads to increased LB formation and suggest a common pathway for the formation of LBs in both dopaminergic and nondopaminergic neurons. The normal levels of these proteins in AD suggest some disease specificity for the significant reduction in chaperone-mediated autophagy proteins and, given the expected partial decrease with age, might suggest some limited upregulation in AD. The labeling of LB inclusions with LC3, and to a lesser extent with LAMP2A and hsc70, provides further support for the involvement of autophagy-related proteins in α-synuclein aggregation. Specifically, the colocalization of LC3 with LBs and small punctate synuclein inclusions is consistent with a role for autophagic vacuoles in the formation of these pro-
tein aggregates. Lysosomal components, including LAMP1, hsc70, and cathepsin D, were observed in LB and were significantly reduced in nigral neurons containing α-synuclein inclusions in PD brains. These results further support the view that impaired lysosomal function may contribute to PD pathogenesis.

We examined the role of decreased chaperone-mediated autophagy protein expression on α-synuclein accumulation using dopaminergic cell cultures and showed that WT α-synuclein is preferentially degraded via chaperone-mediated autophagy rather than the UPS or macroautophagy. These results are in agreement with observations in isolated lysosomes and neuronal cells. Moreover, we have shown that chaperone-mediated autophagy downregulation results in an accumulation of α-synuclein protein. In an earlier report, UPS and macroautophagy were suggested to play an important role in α-synuclein turnover. In agreement with other studies, we were unable to observe a role for the UPS or macroautophagy in α-synuclein degradation. However, under conditions in which chaperone-mediated autophagy was impaired, α-synuclein degradation increasingly involved macroautophagy. A53T α-synuclein expression decreased the turnover of another chaperone-mediated autophagy–regulated protein (GAPDH), consistent with the inhibition of chaperone-mediated autophagy. This is in keeping with our observation that A53T α-synuclein turnover did not involve chaperone-mediated autophagy, but was more dependent on macroautophagy, which supports a recent report using primary neurons and differentiated SH-SYSY cells.

Chaperone-mediated autophagy impairment increased α-synuclein levels and caused markedly impaired cell proliferation, though there was no overt cell death. This was not seen in control cells, suggesting that the increase in WT α-synuclein levels resulted in compromised cell function.

The finding of a specific reduction of chaperone-mediated autophagy proteins in the PD brain adds a further dimension to the pathogenesis of PD and to α-synuclein accumulation and LB formation in particular. We cannot determine whether the chaperone-mediated autophagy changes are primary or secondary to the specific biochemical defects associated with PD. However, a decrease in chaperone-mediated autophagy proteins will increase the half-life of proteins degraded by this system, including α-synuclein, and will involve a cycle of events that promote protein accumulation and aggregation. This reduction will be further enhanced by the age-related decline in chaperone-mediated autophagy activity. In cell models at least, we can conclude that increased α-synuclein levels do not cause decreased LAMP2A levels. The increase in oxidative stress in PD substantia nigra and the resulting accumulation of oxidized protein would normally be expected to increase chaperone-mediated autophagy activity. Thus, the reduction in chaperone-mediated autophagy that we have observed in the PD brain, in addition to the age-related decline in chaperone-mediated autophagy and the increased substrate load of oxidized protein, will together promote accumulation and aggregation of protein in the substantia nigra pars compacta. The finding of reduced chaperone-mediated autophagy protein expression in the amygda provides a possible mechanism for the development of LB pathology outside the substantia nigra pars compacta, though other factors must be required to lead to neuronal loss. Modulation of chaperone-mediated autophagy function in PD might represent a suitable target for drug intervention to modify the deleterious effects of impaired protein handling. In this context, the autophagy enhancer rapamycin has been shown to ameliorate the effects of PINK1 and parkin gene disruption, mitochondrial neurotoxins such as rotenone, and UPS impairment.

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