Majd *et al*. IRHD Journal *(2023) 1:1*

Australian Open Access Journal

DOI:http://doi.org/10.5281/zenodo.10685665

Integrated Research in Health and Disease

ISSN:2981-9229

ORIGINAL RESEARCH **Open Access**

Association of Peroxiredoxin IV with α-synuclein in Neuronal ER Indicates Lewy Bodies Are Seeded within ER in Parkinson's Disease and Dementia with Lewy Bodies.

Laura J Turnbull[1], John H Power[1], Shohreh Majd[1*]

Abstract

Background: Peroxiredoxin IV is a 2-Cys member of the peroxiredoxin antioxidant family that inactivates hydrogen peroxide and is reported to be confined to endoplasmic reticulum (ER) in cultured cell lines. **Methods & Results:** Using human brain tissue from control, Parkinson's disease (PD), and dementia with Lewy bodies (DLB) cases we showed that peroxiredoxin IV is a 26kD protein under reducing conditions in gray and white matter. Using confocal immunohistochemistry, it is most abundant in neurons with lower levels in oligodendrocytes. Peroxiredoxin IV was detected in some microglia that may be due to phagocytosis of neuronal tissue, but it was not detected in astrocytes. Peroxiredoxin IV co-localized with the ER marker Sar1 indicating that it is confined to the ER. Co-localization of peroxiredoxin IV with α-synuclein in PD substantia nigra and DLB cortex indicated that α-synuclein aggregation was seeded within ER and that mature Lewy bodies were closely associated with the ER. In PD there was a more complex interaction between ER, α-synuclein and the DNA dye dapi suggesting some nuclear degradation in PD. **Conclusion:** These results show that peroxiredoxin IV is primarily a neuronal protein that is confined to ER. In addition, we show that α-synuclein aggregation and Lewy body development is seeded within ER.

Keywords: Peroxiredoxin IV, Substantia nigra, ER trafficking, Sar1, Alpha-synuclein, Parkinson's disease, Dementia with Lewy bodies, Neurons, Oligodendrocytes

Introduction

Peroxiredoxin IV was originally identified as a member of the alkyl hydroperoxide reductase/thiol-specific antioxidant super family of proteins based on sequence homology (Chae et al. 1994). Peroxiredoxin IV is now more specifically defined as a member of the peroxiredoxin family of antioxidant enzymes. The family consists of six members characterized by a redox active cysteine residue that detoxifies peroxides that becomes oxidized in the process to cysteine sulfenic acid. Peroxiredoxin I to IV are referred to as typical 2-Cys peroxiredoxins and function as homodimers. Peroxiredoxin V is functionally monomeric and is referred to an atypical 2-Cys peroxiredoxin and peroxiredoxin VI is the 1-Cys member of the family (Wood et al. 2003). Peroxiredoxin IV is the least characterized of the peroxiredoxin family with some recent studies on its role in cancer (Rafiei et al. 2015). Previous studies described Peroxiredoxin IV as a thioredoxin peroxidase and termed

∗ Corresponding author. [1]Physiology Department, Flinders Medical Centre, Level 6, Rm 6E404 Bedford Park, SA 5042, Australia. E-mail addresses: shohreh.majd@flinders.edu.au (S. Majd), jhtp@protonmail.com (J.H. Power), lauraturnbull24@gmail.com (L.J. Turnbull).

antioxidant enzyme AOE372. The label AOE372 defines a redox pathway that leads to NF-κB activation. Using a yeast two hybrid approach Jin et al isolated a cDNA as a Pag partner that encoded peroxiredoxin IV (Jin et al. 1997). Haridas and co-workers identified the same enzyme by sequence comparison with expressed sequence tag databases and by functional screening assays. This was termed as TRANK (thioredoxin peroxidase-related activator of NF-κB and c-Jun N-terminal kinase), which was considered to be a secreted protein with a role in inflammation (Haridas et al. 1998; Schulte 2011).

Peroxiredoxin IV is different from other 2-Cys members in that it contains a hydrophobic leader which suggests that peroxiredoxin IV is a secreted protein. Using pulse-chase experiments with peroxiredoxin IV transfected COS-1 cells it was reported that peroxiredoxin IV is processed and released from cells within 10 mins (Matsumoto et al. 1999; Okado-Matsumoto et al. 2000; Tahmasbpour Marzony et al. 2016). While Kakihana et al, suggested the presence of peroxiredoxin IV in early secretory compartments of cells (Kakihana et al. 2013), the idea that peroxiredoxin IV is secreted was questioned by some other studies. Using pulse-chase experiments on HT1080 cells and HT1080 cells stably transfected to overexpress peroxiredoxin IV Tavender and colleagues concluded that peroxiredoxin IV is retained within the endoplasmic reticulum (ER) and that over-expression did not induce ER stress (Tavender et al. 2008). Furthermore, while treatment with hydrogen peroxide did compromise peroxiredoxin IV knockdown cells, this did not occur in a dose dependant manner, and they have suggested that the role of peroxiredoxin IV in the ER maybe to act as a molecular chaperone. Supporting this concept was the finding that sucrose gradient analyses of whole cell extracts indicated that peroxiredoxin IV formed stable oligomers which diminished its ability to act as a peroxidase. It was further reported by Wang and co-workers using crystal structure determination of E-coli expressed peroxiredoxin IV that it exists in its native form as a decamer comprising five catalytic dimers (Wang et al. 2012).

The fact that peroxiredoxin IV is confined to the ER, can act as an antioxidant and possibly as a molecular chaperone could implicate this enzyme as having a potentially important role in neurons that experience ER stress as occurs in a number of neurodegenerative diseases.

There are several reports using a variety of models suggesting that mutant or modified α-synuclein can induce the unfolded protein response within the ER and cell death (Smith et al. 2005; Sugeno et al. 2008). In addition, in yeast models it has been reported that α-synuclein blocks ER vesicles from docking with the Golgi apparatus resulting vesicular accumulation (Cooper et al. 2006; Kincaid and Cooper 2007).

To reveal the localization of peroxiredoxin IV, here we examined the cellular distribution of peroxiredoxin IV in the human brain and its association with Lewy body pathology in Parkinson's disease (PD) and dementia with Lewy bodies (DLB).

Methods

Antibodies

Primary antibodies. Peroxiredoxin IV (rabbit, ab59542), Sar1 (chicken, ab14270), Abcam plc, Cambridge UK, α-synuclein (sheep, AS3SB), Antibody Technology Australia Pty, Ltd, Adelaide, Australia. Glial fibrillary acidic protein (GFAP) (mouse, G5601) Novacastra Laboratories Ltd, Newcastle, UK.

Human neuronal protein HuC/HuD (mouse, A-21271) Molecular Probes Inc, Eugene, USA. Human HLA (MHC2) (mouse, M0775, clone CR3/43) Dakocytomation, Glostrup, Denmark. Myelin basic protein (MBP) (mouse, NCL-MBP, clone 7H11), Novacastra Laboratories Ltd, Newcastle, UK.

Secondary antibodies. Donkey anti chicken Cy2 (703-225-152), donkey anti mouse Cy2 (715-225-150), donkey anti rabbit Cy3 (711-165-152), donkey anti sheep DyLight 488 (713-485-147), Jackson Immunoresearch, West Grove, USA. Donkey anti rabbit alkaline phosphatase (A-3687), Sigma Chemical Company, St Louis, USA.

Brain homogenates and sample preparation

The brain tissue was obtained with Flinders University Human Ethic clearance from the National Health and Medical Research Council South Australian Brain Bank. PD and DLB cases were diagnosed using the revised criteria for the clinical and pathologic diagnosis. The control cases were obtained from people who died of unrelated causes without diagnosed neurological disease and absence of LB pathology confirmed by neuropathological examination. Frozen tissue from middle frontal gyrus from a PD, DLB and control case were carefully dissected into white and gray matter and homogenized in 50mM Tris, 5mM EDTA, 0.02% thimerosal, 1µl/ml Pepstatin and Leupeptin and 0.3mM phenylmethylsulfonyl fluoride (Table 1). Five ml of homogenization buffer was used per gram of brain tissue and homogenized using six passes of a motorized Wheaton Teflon pestle tissue grinder. Homogenates were centrifuged (200 x g) for 15 min to remove particulate matter and snap frozen at -80°C. The details of these cases are listed in table 1. Samples were assayed for total protein and prepared in non-reducing and reducing sample (+DTT) buffer at a concentration of 2µg/µl. All samples were boiled for 10 min just prior to electrophoresis and 20µg of protein was loaded per well. In some experiments the samples were reduced for periods up to 48h at 37°C.

Case Number	Sex	Age (years)	Diagnosis	Region	PMI (hour)
C1	M	72	NFA	MFG	30
PD1	F	85	PD	MFG	12
DLB1	F	95	DLB	MFG	9

M= Male, *F*= Female, *NFA*= Normal for age, *C*= Control, *PD*= Parkinson's disease, DLB= Dementia with Lewy body disease, M*FG*= Middle frontal gyrus, *PMI*= Post mortem interval

Table 1. List of cases used for 1D PAGE and Western blots.

One-dimensional electrophoresis and Western blotting

Proteins from brain homogenates were separated using a Bio-Rad Mini gel System (Bio-Rad, Richmond, USA) and 12% polyacrylamide gels. The separated proteins were transferred onto PVDF membrane (BioRad, Immun-Blot, Cat # 162-0177) using a Semidry Transfer Unit (model TE70

SemiPhor; Hoefer Inc, Holliston, USA) using a transfer buffer of 0.25 mol/L Tris, 0.192 mol/L glycine, and 20% methanol for 90 minutes at 0.8mA/cm^2. The PVDF membrane containing the transferred proteins was blocked with milk proteins

Immunohistochemistry

Light Microscopy

Parkinson's disease, DLB and control brain sections detailed in table 2, were deparaffinized, subjected to EDTA antigen retrieval, treated with hydrogen peroxide and blocked with normal horse serum and then incubated for 18h with either rabbit peroxiredoxin IV antibodies or pre-immune serum as a negative control. The primary antibodies were visualized with biotinylated goat anti-rabbit secondary antibodies (Sigma) and the antibody complex visualized using a Vector ABC kit (Vector Laboratories Inc., Burlingame, USA) and DAB substrate (Sigma). Sections were examined and photographed with an Olympus BX50 microscope linked to a QImaging Micro Publisher 5.0 RTV camera (Q Imaging, Surrey, Canada*)*.

Table 2. List of cases used in light and confocal immunohistochemistry.

Case Number	Sex	Age (yr)	Diagnosis	Region	PMI (hr)
C2	M	72	NFA	CG/MFG	30
C3	F	86	NFA	CG/MFG	25
DLB2	F	81	DLB	CG/MFG	7
DLB3	M	80	DLB	CG/MFG	6
DLB4	F	95	DLB	CG/MFG	9
PD2	M	79	DLB/PD	CG/MFG/SN	24
PD3	F	76	PD	CG/MFG/SN	19
PD4	F	91	PD	CG/MFG/SN	24

M male, *F* female, *NFA* Normal for age, *C* Control, *PD* Parkinson's disease, *DLB* dementia with Lewy body disease, M*FG* Middle frontal gyrus, *CG* Cingulate gyrus, *SN* Substantia Nigra, *PMI* postmortem interval.

Cellular localization of Peroxiredoxin IV

Parkinson's disease, DLB and control brain sections (table 2) were deparaffinized, subjected to EDTA antigen retrieval then blocked with normal horse (5%) in PBS and incubated overnight with a primary antibody to peroxiredoxin IV. The antigen-antibody complex was visualized using alkaline phosphatase and BCIP/NBT substrate tablets (Sigma, Cat # B-5655).

serum and then incubated for 18h with rabbit antibodies against peroxiredoxin IV and antibodies against cellular markers as previously described (Power et al. 2002). The primary antibodies were visualized using secondary antibodies conjugated to the following fluorescent fluorophores, donkey anti mouse Cy2 (Jackson) and donkey anti rabbit Cy3 (Jackson). The cellular distribution of peroxiredoxin IV was compared with the staining obtained with antibodies to specific cellular markers. Antibodies to GFAP (astrocytes), HLA (MHC2) (microglia), MBP (oligodendrocytes) and HuC/HuD (neurons) were used to map the distribution of peroxiredoxin IV in human tissue. Sections were treated with the nuclear stain DAPI followed by 1% Sudan black (Sigma, S0395) in 70% ethanol for 5 min to block endogenous florescence. Sections were examined using a Bio-Rad Confocal laser scanning microscope and Bio-Rad software package or a Leica SP5 Confocal laser scanning microscope.

Colocalization of peroxiredoxin IV with Sar1 (ER marker) and α-synuclein (Lewy body pathology)

Parkinson's disease and DLB sections containing Lewy bodies were processed as described above and incubated with rabbit antibodies against peroxiredoxin IV, chicken antibodies against Sar1 and sheep antibodies to α-synuclein. The primary antibodies were visualized using secondary antibodies previously listed.

Results

Characterisation of the peroxiredoxin IV antibody in human brain tissue

Human brain proteins (20μg per well) from PD, DLB and control tissue were separated under non reducing and reducing conditions using PAGE and

stained with Coomassie blue as shown in figure 1a and 1b. Identical gels were transferred to PVDF and probed with a peroxiredoxin IV antibody raised in rabbits to a peptide of human peroxiredoxin IV (Abcam).

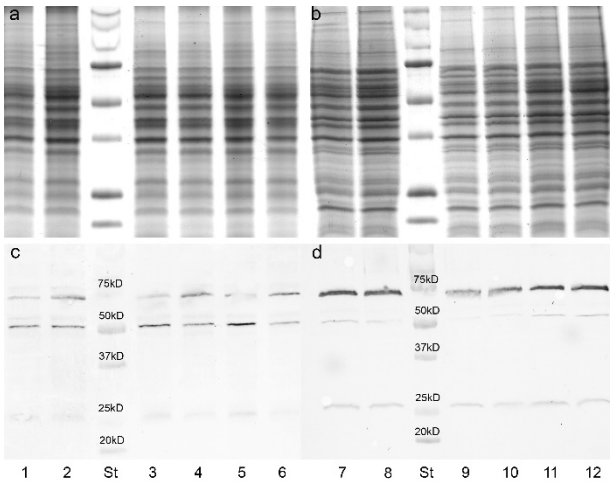

Fig 1. Coomassie blue stained PAGE gel (a, unreduced and b, reduced) and corresponding blot (c, unreduced and d, reduced) of repeat gel probed with peroxiredoxin IV antibodies of white and gray matter from control (C1) , PD (PD1) and DLB (DLB1) tissue. Lanes 1-6 unreduced. Lane 1, C, MFG, WM; 2, C, MFG, GM; 3, PD, MFG, WM; 4, PD, MFG, GM; 5, DLB, MFG, WM; 6, DLB, MFG, GM. Lanes 7-12 reduced. Lane 7, C, MFG, WM; 8, C, MFG, GM; 9, PD, MFG, WM; 10, PD, MFG, GM; 11, DLB, MFG, WM; 12, DLB, MFG, GM. *St* Standards in kD. C control, PD Parkinson's disease, DLB dementia with Lewy bodies, MFG middle frontal gyrus, WM white matter, GM gray matter.

Western blotting of unreduced gels indicated that peroxiredoxin IV was present as a range of proteins. Unreduced blots indicated that the monomer was present as a faint band at about 26kD. The dimer was more prominent at 52kD. Two faint bands just above and below the dimer were also visible. Another band was also visible at a similar intensity to the dimer just below the 75kD standard at approximately 73kD (Fig. 1c). A few other faint bands at higher molecular weight were visible on the blots but did not reproduce well. Under reducing conditions only three bands were visible, the monomer at 26kD, the dimer at 52kD and the band at 73kD (Fig. 1d).

Under these conditions the 73kD band was the most prominent band followed by the monomer. Despite being reduced the dimer was still visible. It appeared that the higher molecular weight forms reduced down to very stable trimer at 73kD. Further experiments in which the samples were reduced for varying periods up to 48h at 37°C showed that the monomer at 26kD was the major species but faint bands at 52kD and 73kD were still visible (Fig. 2).

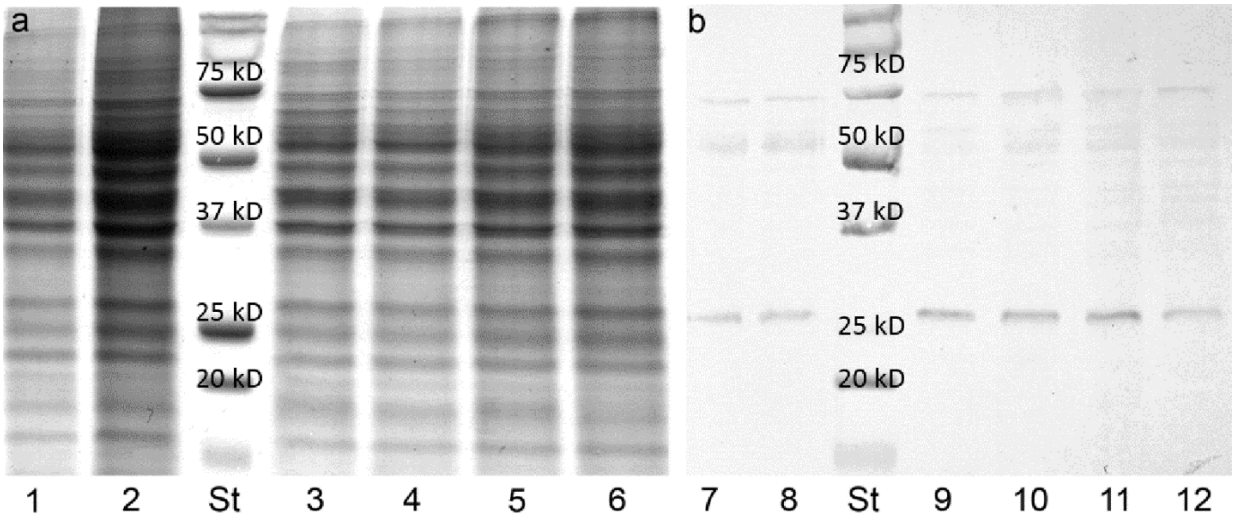

Figure 2) Coomassie blue stained PAGE gel (a) and corresponding blot (b) of repeat gel of homogenates samples from white and gray matter from control (C1), PD (PD1) and DLB (DLB1). Samples were reduced for 48h at 37°C and the blot probed with peroxiredoxin IV antibodies. Lane 1, C, MFG, WM; 2, C, MFG, GM; 3, PD, MFG, WM; 4, PD, MFG, GM; 5, DLB, MFG, WM; 6, DLB, MFG, GM; 7, C, MFG, WM; 8, C, MFG, GM; 9, PD, MFG, WM; 10, PD, MFG, GM; 11, DLB, MFG, WM; 12, DLB, MFG, GM. *St* Standards in kD. C control, PD Parkinson's disease, DLB dementia with Lewy bodies, MFG middle frontal gyrus, WM white matter, GM gray matter.

Localisation of peroxiredoxin IV in human brain tissue

Using immunohistochemistry at the light microscopy level with peroxiredoxin IV antibodies and DAB staining we have shown that neurons have the strongest staining with some staining in glial cells. Staining was in the cytoplasm and had a vesicular appearance.

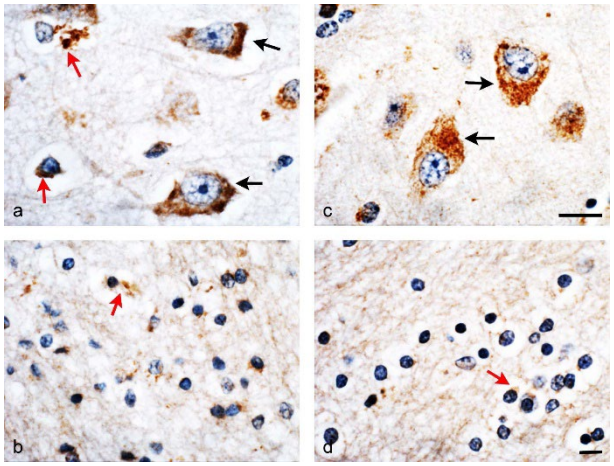

Fig 3. Light immunohistochemistry using peroxiredoxin IV antibodies (1/250) (DAB substrate) at oil immersion (1000X) showing typical staining of neurons (black arrows) and oligodendrocytes (red arrows) seen in DLB and control tissue. Figure a, control gray matter; figure b, control white matter; figure c, DLB gray matter; figure d, DLB white matter. Red arrows indicate staining surrounding the nucleus of oligodendrocytes (bar = 10μM).

It was not evenly distributed, and some cytoplasmic regions were more intensely stained than others. Oligodendrocytes were the glial cells with the most obvious staining but were not as intensely stained as neurons (Fig. 3).

Cellular distribution of peroxiredoxin IV in human brain tissue

Using immunohistochemical markers for specific human brain cell types we have shown that peroxiredoxin IV is present in neurons in PD, DLB and in control tissue (Fig. 4a-c, DLB shown). Peroxiredoxin IV was not detected in astrocytes (Fig. 4d-f) and did not co-localize with MBP (Fig. 4j-l). However light immunohistochemistry with PIV in figure 3 clearly indicated that many but not all oligodendrocytes had some staining around the nucleus. The majority of microglia as stained by the MHC2 antibody did not contain peroxiredoxin IV. Some microglia were positive as shown in figure 4g-i and some were observed surrounding and internalizing "clumps" of peroxiredoxin IV (data not shown) which we suggest are parts of dead neurons that were in the process of being ingested.

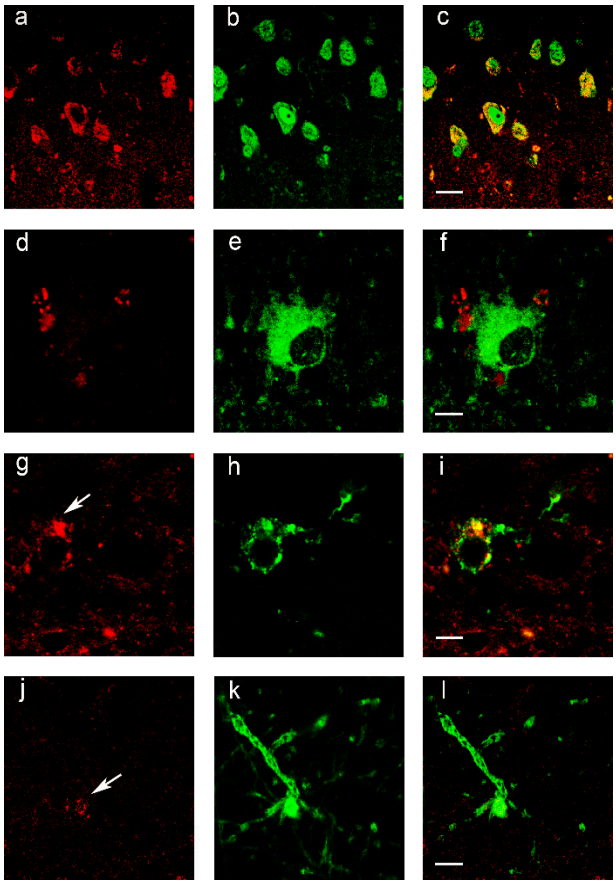

Fig. 4. Confocal localization of peroxiredoxin IV (1/200) with Hu (1/100, neuronal marker) (a, peroxiredoxin IV, Cy3-red; b, Hu, Cy2-green; c, merged image; bar = 10μM). GFAP (1/100, astrocyte marker) (d, peroxiredoxin IV, Cy3-red; e, GFAP Cy2-green; f, merged image; bar = 20μM). MHC2 (1/100, microglia marker) (g, peroxiredoxin IV, Cy3-red; h, MHC2, Cy2-green; i, merged image; bar = 5μM). MBP (1/100, myelin marker) (j, peroxiredoxin IV, Cy3-red; k, MBP, Cy2-green; l, merged image; bar = 10μM). Panels a-c, DLB, middle frontal gyrus, gray matter; d-f, DLB, middle frontal gyrus, gray matter; g-i, DLB, middle frontal gyrus, white matter; j-l, DLB, middle frontal gyrus, white matter.

Colocalization of peroxiredoxin IV and Sar1

Peroxiredoxin IV co-localised with the ER-Golgi marker Sar1 indicating that peroxiredoxin IV was localized to the ER (Fig. 5).

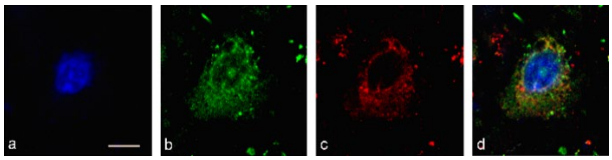

Fig. 5. Confocal localization of peroxiredoxin IV (DLB3, MFG) (1/200) (c, Cy3-red) with DAPI (neuronal dye) (a, blue); Sar1 (1/100, Endoplasmic reticulum marker) (b, Cy2-green); merged image (d); bar = 10μM).

Labels for both proteins surrounded the neuronal nucleus but at higher magnification it was apparent that both proteins are co-localized in the same organelle. Sar1 appeared to have a slightly wider distribution than peroxiredoxin IV which is consistent with Sar1 being involved with Golgi apparatus trafficking vesicles.

Co-localization of peroxiredoxin IV and α-synuclein in DLB

In DLB tissue α-synuclein first appeared in neurons as small aggregations within the ER.

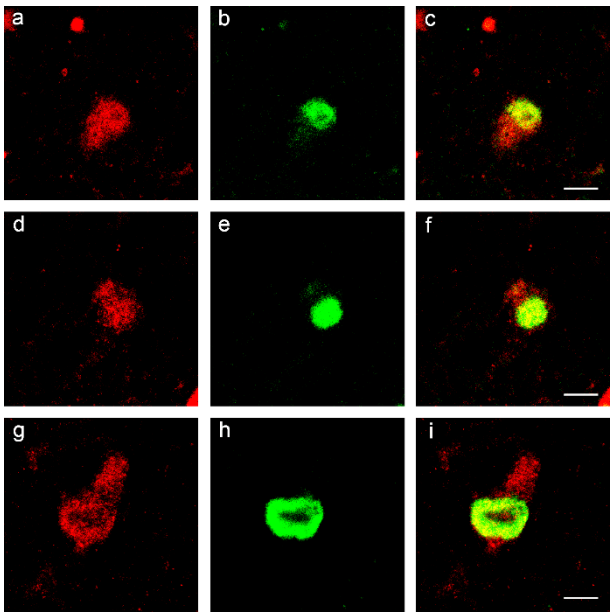

Fig. 6. Confocal localization of peroxiredoxin IV (1/200) (Cy3-red) and α-synuclein (1/250) (DyLight 488-green) in neurons containing aggregations of α-synuclein within the endoplasmic reticulum in dementia with Lewy bodies disease. (peroxiredoxin IV a,d,g; α-synuclein b,e,h; merged image c,f,i, bar = 10μM).

The α-synuclein label was seen as deposits within the peroxiredoxin IV staining. There was a spectrum of aggregations of different sizes observed within the ER and as the α-synuclein aggregations enlarged and developed into concentric Lewy bodies they were still associated with the ER (Fig. 6).In larger unstructured Lewy bodies peroxiredoxin IV was seen to be evenly distributed throughout the Lewy body and colocalized with α-synuclein. In concentric Lewy bodies peroxiredoxin IV was partially colocalized with α-synuclein but was more concentrated in the core and around the periphery of the Lewy body and not colocalized with α-synuclein (Fig. 7).

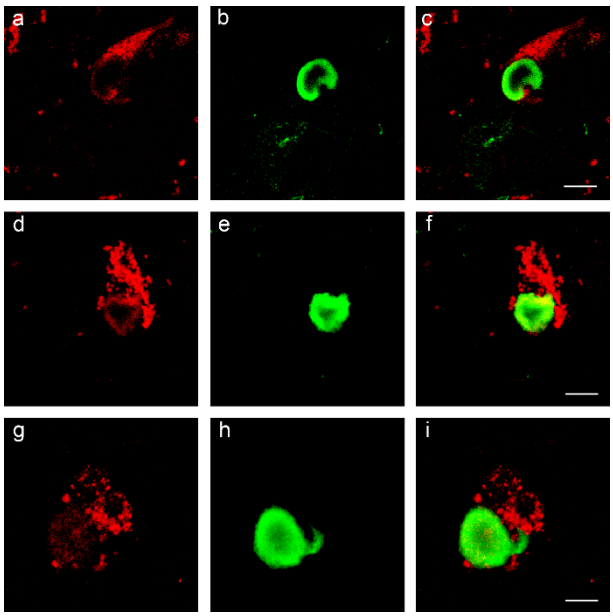

Fig. 7. Confocal localization of peroxiredoxin IV (1/200) (Cy3-red) and α-synuclein (1/250) (DyLight 488-green) in neurons containing Lewy bodies showing the close association with the endoplasmic reticulum in dementia with Lewy bodies disease. (peroxiredoxin IV a,d,g; α-synuclein b,e,h; merged image c,f,i, bar = 10μM).

Colocalization of peroxiredoxin IV and α-synuclein in PD

In PD substantia nigra tissue small α-synuclein aggregations were seen to be seeded within the ER. At this stage the neuronal nucleus remained structured and intact (Fig. 8a-d). As these aggregations developed a range of structures were observed. In some cells α-synuclein developed as a

defined ring enclosing diffuse dapi staining surrounded or associated with the ER (Fig. 8e-l). In other cells a range of more complex interactions were seen with multiple aggregations of α-synuclein enclosing dapi staining associated and co-localised with the ER (Fig. 8m-p).

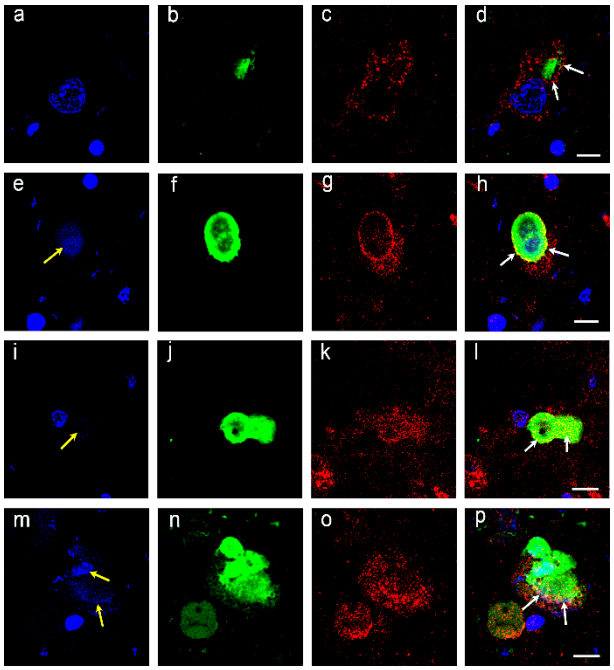

Fig. 8. Confocal localization of the nuclear dye DAPI (blue), peroxiredoxin IV (1/200) (Cy3-red) and α-synuclein (1/250) (DyLight 488-green) in neurons containing α-synuclein aggregations and Lewy bodies showing the close association with the endoplasmic reticulum in the substantia nigra in Parkinson's disease. (DAPI a,e,i,m; peroxiredoxin IV b,f,j,m; α-synuclein c,g,k,o; merged images d,h,i,p, bar = 10μM).

Discussion

We have shown peroxiredoxin IV to be a 26kD molecular weight protein in its reduced form that is confined to the ER and is predominately localized to neurons in human brain tissue. This protein is unusual in that it is synthesised with a signal peptide yet still appears to be retained within the ER which is consistent with previously published reports (Tavender et al. 2008; Yang et al. 2016). Western blots of unreduced human proteins from gray and white brain tissue from PD, DLB and control tissue showed a monomer of approximately 26kD, a dimer of 52kD and a series of faint higher molecular weight bands consistent with the formation of multimers up to pentamers as seen in cell culture. A protein of similar intensity to the dimer was also seen at approximately 73kD. Under reducing conditions only three proteins were detected. The 26kD monomer was more prominent than the 52kD dimer indicating that the dimer had not been completely reduced. The most intensely stained protein was at 73kD.

It has previously been suggested that this protein functions as an antioxidant and in its aggregated forms as a molecular chaperone. Under both reduced and unreduced conditions the monomer and dimer are visible, but the intensities are reversed. The 73kD species was greatly intensified when the samples were reduced which we suggest is a trimer. The trimer was a very stable species, and only when homogenates were incubated at 37°C for 48h were we able to reduce the 73kD species and even then a trace remained. A trimer has not previously been reported and is probably the result of the sample preparation conditions. It was most abundant after reduction, while there are also evidence indicating that this enzyme is organised as five dimer subunits making up a decamer in its native form (Wang et al. 2012).

This protein functions as an antioxidant as a concomitant dimer (Morais et al. 2015) and we have found that in human brain tissue the dimer re-forms after being reduced. The monomer is observed in freshly reduced preparations, but the dimer is progressively observed when the same sample is stored. Tavender and co-workers have indicated that a strong reducing system is present in the ER to recycle the disulphide formation as a novel pathway (Tavender and Bulleid 2010; Tavender et al. 2010).

The full length of this protein contains 271 amino acids with a predicted molecular weight of 30.5kD. The 30.5kD protein was not detected in our brain samples, however without the 37 amino acid signal peptide this protein has a predicted molecular weight of approximately 26.3kD.

Our findings from light and confocal immunohistochemistry using peroxiredoxin IV antibody and specific cellular markers demonstrated that this protein was primarily localized in cytoplasm of neurons. It was not present in astrocytes and did not co-localize with myelin basic protein. Peroxiredoxin IV was present as deposits around the oligodendrocyte nucleus in the gray matter in our light microscopy results. Only a few oligodendrocytes in the white matter showed similar staining around the nucleus. Peroxiredoxin IV was present in some but not all microglia which suggests it might be present in a subpopulation of microglia or may in fact have been phagocytosed neuronal debris that contained peroxiredoxin IV. A similar cellular distribution has been reported in mouse brain but with a stronger staining in oligodendrocytes (Jin et al. 2005). The histology indicated that in neurons, peroxiredoxin IV was confined to a particular region of the cytoplasm supporting the localisation to the ER. This was confirmed by co-localisation of peroxiredoxin IV with the ER/Golgi protein Sar1. Sar1 is a member of the small GTPase super family and is involved in vesicular transport from the ER to the Golgi apparatus (Ackema et al. 2016) . Although there was considerable co-localization, Sar1 appeared to have a wider distribution than peroxiredoxin IV which is consistent with it being confined to the ER while Sar1 is also associated with the Golgi complex (Yavuz and Warren 2017).

At a cellular level the defining pathology of PD and DLB is the presence of Lewy bodies in the substantia nigra and cortical neurons, respectively (Tsuboi and Dickson 2005). The mechanism by which Lewy bodies develop and grow is unresolved but recent findings using the expression of α-synuclein in a yeast model have suggested that α-synuclein blocks the docking of transport vesicles from the ER to the Golgi apparatus and these vesicles accumulate (Cooper et al. 2006). Our current study on human brain tissue supports this concept and we suggest that Lewy bodies are seeded within the ER. Although the exact structure is beyond the resolution of the confocal microscope in some neurons small vesicles that were positive for both α-synuclein and peroxiredoxin IV were seen aggregating within the ER around the neuronal nucleus. The peroxiredoxin IV staining was very granular while the α-synuclein was much more diffuse. At this level it would only be conjecture as to whether there is any relationship between α-synuclein and peroxiredoxin IV. Peroxiredoxin IV is confined to the ER and α-synuclein is synthesised and modified within in the ER possibly as a result of ER stress, which were proven previously (Hetz and Mollereau 2014; Mercado et al. 2016). Alternatively, oxidized or modified cytosolic α-synuclein is associating with ER trafficking vesicles bound for the Golgi. In yeast models, α-synuclein is a foreign protein and the ER-Golgi complex is not designed to handle α-synuclein but in human disease the α-synuclein must be modified in some way otherwise all cells would develop Lewy bodies. As part of the disease process, it is likely that α-synuclein has been oxidized due to increased oxidative stress which is elevated in PD and DLB. It has been reported that α-synuclein is a helically folded tetramer that resists aggregation (Bartels et al. 2011). It is likely that this structure has been modified in PD and DLB and results in miss-handling by the ER-Golgi complex and the aggregation we have described. Alternatively, α-synuclein could be oxidized in the cytosol resulting in a breakdown of the tetramer and the monomer being inserted into vesicles bound for the Golgi complex.

As Lewy bodies develop, they progress from a diffuse structure as seen in figure 6 to become more organised with concentric rings of specific proteins (Gai et al. 2000). Alpha synuclein is one of the outer rings giving a mature Lewy body a circular structure when IT stained with an α-synuclein antibody in our study. The advanced or mature Lewy bodies still showed a slight co-localisation with peroxiredoxin

IV but the majority of staining was distinct. We showed that mature or concentric Lewy bodies were still closely associated with the ER and looked to be fed by material from the ER. Lewy bodies comprise of many proteins from a variety of cellular components (Xia et al. 2008), and it is likely that once the formation of a Lewy body is initiated, it develops the ability to recruit other cellular components as many vesicular and mitochondrial fragments are also seen at the EM level (Gai et al. 2000; Gallegos et al. 2015).

There are notable differences between the development of Lewy bodies in neurons in the cortex in DLB and in the substantia nigra in PD (Kim et al. 2014). In DLB there appears to be a regular progression from α-synuclein aggregation within the ER to a more condensed Lewy body still associated with the ER as we observed in our results. In DLB, neurons only develop one Lewy body, while in PD the profile is quite different with a much more diverse range of α-synuclein aggregations. In the substantia nigra α-synuclein aggregation begins as a diffuse structure surrounded by the ER, as we showed in our findings (Fig. 8). In some neurons the α-synuclein staining appears as a number of tight rings indicating multiple small Lewy bodies (data not shown). Many Lewy bodies in the substantia nigra have a discrete ring structure when stained for α-synuclein with strong diffuse DAPI staining surrounded by the ER indicating nuclear degradation (Fig. 8h). Other Lewy bodies have regions of diffuse α-synuclein aggregation associated with a defined ring of α-synuclein staining as shown in figure 8l and 8p. It appeared that the α-synuclein aggregation was involved with nuclear degradation and the released DNA was interacting with the α-synuclein producing two distinct structures. The DNA staining seen in Lewy bodies was diffuse and homogenous and quite different from normal nuclear staining as seen in figure 8d and probably indicates that the process of apoptosis has commenced. These observations suggest that although there are similar processes involved in the formation of DLB and PD Lewy bodies there appears to be additional more complex mechanisms involving nuclear degradation in PD.

Conclusion

In conclusion, we suggest that peroxiredoxin IV is primarily a neuronal protein in human brain which is localised to the ER. Using peroxiredoxin IV as a marker for the ER we have shown that Lewy bodies appear to be seeded within the ER and as they develop, they remain associated with the ER, and there are considerable differences between DLB and PD Lewy bodies. Further investigation is required to determine the differences between Lewy bodies in PD and DLB.

Acknowledgments

We gratefully acknowledge the excellent assistance of the Flinders University Microscopy and Image Analysis Facility and the South Australian Brain Bank. We would like to thank Professor Peter Blumbergs for reading this manuscript and his constructive comments. This work was supported by grants from the Flinders Medical Centre Foundation and Foundation Daw Park.

References

Ackema KB, Prescianotto-Baschong C, Hench J, Wang SC, Chia ZH, Mergentaler H, Bard F, Frank S, Spang A (2016) Sar1, a Novel Regulator of ER-Mitochondrial Contact Sites. PLoS One 11:e0154280

Bartels T, Choi JG, Selkoe DJ (2011) alpha-Synuclein occurs physiologically as a helically folded tetramer that resists aggregation. Nature 477:107-110

Chae HZ, Robison K, Poole LB, Church G, Storz G, Rhee SG (1994) Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. Proc Natl Acad Sci U S A 91:7017-7021

Cooper AA, Gitler AD, Cashikar A, Haynes CM, Hill KJ, Bhullar B, Liu K, Xu K, Strathearn KE, Liu F, Cao S, Caldwell KA, Caldwell GA, Marsischky G, Kolodner RD, Labaer J, Rochet JC, Bonini NM, Lindquist S (2006) Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models. Science 313:324-328

Gai WP, Yuan HX, Li XQ, Power JT, Blumbergs PC, Jensen PH (2000) In situ and in vitro study of colocalization and segregation of alpha-synuclein, ubiquitin, and lipids in Lewy bodies. Exp Neurol 166:324-333

Gallegos S, Pacheco C, Peters C, Opazo CM, Aguayo LG (2015) Features of alpha-synuclein that could explain the progression and irreversibility of Parkinson's disease. Front Neurosci 9:59

Haridas V, Ni J, Meager A, Su J, Yu GL, Zhai Y, Kyaw H, Akama KT, Hu J, Van Eldik LJ, Aggarwal BB (1998) TRANK, a novel cytokine that activates NF-kappa B and c-Jun N-terminal kinase. J Immunol 161:1-6

Hetz C, Mollereau B (2014) Disturbance of endoplasmic reticulum proteostasis in neurodegenerative diseases. Nat Rev Neurosci 15:233-249

Jin DY, Chae HZ, Rhee SG, Jeang KT (1997) Regulatory role for a novel human thioredoxin peroxidase in NF-kappaB activation. J Biol Chem 272:30952-30961

Jin MH, Lee YH, Kim JM, Sun HN, Moon EY, Shong MH, Kim SU, Lee SH, Lee TH, Yu DY, Lee DS (2005) Characterization of neural cell types expressing peroxiredoxins in mouse brain. Neurosci Lett 381:252-257

Kakihana T, Araki K, Vavassori S, Iemura S, Cortini M, Fagioli C, Natsume T, Sitia R, Nagata K (2013) Dynamic regulation of Ero1alpha and peroxiredoxin 4 localization in the secretory pathway. J Biol Chem 288:29586-29594

Kim WS, Kagedal K, Halliday GM (2014) Alpha-synuclein biology in Lewy body diseases. Alzheimers Res Ther 6:73

Kincaid MM, Cooper AA (2007) Misfolded proteins traffic from the endoplasmic reticulum (ER) due to ER export signals. Mol Biol Cell 18:455-463

Matsumoto A, Okado A, Fujii T, Fujii J, Egashira M, Niikawa N, Taniguchi N (1999) Cloning of the peroxiredoxin gene family in rats and characterization of the fourth member. FEBS Lett 443:246-250

Mercado G, Castillo V, Soto P, Sidhu A (2016) ER stress and Parkinson's disease: Pathological inputs that converge into the secretory pathway. Brain Res 1648:626-632

Morais MA, Giuseppe PO, Souza TA, Alegria TG, Oliveira MA, Netto LE, Murakami MT (2015) How pH modulates the dimer-decamer interconversion of 2-Cys peroxiredoxins from the Prx1 subfamily. J Biol Chem 290:8582-8590

Okado-Matsumoto A, Matsumoto A, Fujii J, Taniguchi N (2000) Peroxiredoxin IV is a secretable protein with heparin-binding properties under reduced conditions. J Biochem 127:493-501

Power JH, Shannon JM, Blumbergs PC, Gai WP (2002) Nonselenium glutathione peroxidase in human brain : elevated levels in Parkinson's disease and dementia with lewy bodies. Am J Pathol 161:885-894

Rafiei S, Tiedemann K, Tabaries S, Siegel PM, Komarova SV (2015) Peroxiredoxin 4: a novel secreted mediator of cancer induced osteoclastogenesis. Cancer Lett 361:262-270

Schulte J (2011) Peroxiredoxin 4: a multifunctional biomarker worthy of further exploration. BMC Med 9:137

Smith WW, Jiang H, Pei Z, Tanaka Y, Morita H, Sawa A, Dawson VL, Dawson TM, Ross CA (2005) Endoplasmic reticulum stress and mitochondrial cell death pathways mediate A53T mutant alpha-synuclein-induced toxicity. Hum Mol Genet 14:3801-3811

Sugeno N, Takeda A, Hasegawa T, Kobayashi M, Kikuchi A, Mori F, Wakabayashi K, Itoyama Y (2008) Serine 129 phosphorylation of alpha-synuclein induces unfolded protein response-mediated cell death. J Biol Chem 283:23179-23188

Tahmasbpour Marzony E, Ghanei M, Panahi Y (2016) Oxidative stress and altered expression of peroxiredoxin genes family (PRDXS) and sulfiredoxin-1 (SRXN1) in human lung tissue following exposure to sulfur mustard. Exp Lung Res 42:217-226

Tavender TJ, Bulleid NJ (2010) Peroxiredoxin IV protects cells from oxidative stress by removing H2O2 produced during disulphide formation. J Cell Sci 123:2672-2679

Tavender TJ, Sheppard AM, Bulleid NJ (2008) Peroxiredoxin IV is an endoplasmic reticulum-localized enzyme forming oligomeric complexes in human cells. Biochem J 411:191-199

Tavender TJ, Springate JJ, Bulleid NJ (2010) Recycling of peroxiredoxin IV provides a novel pathway for disulphide formation in the endoplasmic reticulum. Embo j 29:4185-4197

Tsuboi Y, Dickson DW (2005) Dementia with Lewy bodies and Parkinson's disease with dementia: are they different? Parkinsonism Relat Disord 11 Suppl 1:S47-51

Wang X, Wang L, Wang X, Sun F, Wang CC (2012) Structural insights into the peroxidase activity and inactivation of human peroxiredoxin 4. Biochem J 441:113-118

Wood ZA, Schroder E, Robin Harris J, Poole LB (2003) Structure, mechanism and regulation of peroxiredoxins. Trends Biochem Sci 28:32-40

Xia Q, Liao L, Cheng D, Duong DM, Gearing M, Lah JJ, Levey AI, Peng J (2008) Proteomic identification of novel proteins associated with Lewy bodies. Front Biosci 13:3850-3856

Yang K, Li DF, Wang X, Liang J, Sitia R, Wang CC, Wang X (2016) Crystal Structure of the ERp44-Peroxiredoxin 4 Complex Reveals the Molecular Mechanisms of Thiol-Mediated Protein Retention. Structure 24:1755-1765

Yavuz S, Warren G (2017) A role for Sar1 and ARF1 GTPases during Golgi biogenesis in the protozoan parasite Trypanosoma brucei. Mol Biol Cell 28:1782-1791

Conflict of interest

The authors declare no conflicts of interest regarding the content presented in this manuscript.