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Brief communication

Upregulation of alphaB-crystallin expression in the substantia nigra of patients with Parkinson’s disease

Yingjun Liu, Qinbo Zhou, Mi Tang, Ning Fu, Wei Shao, Shuzhen Zhang, Yanqing Yin, Rong Zeng, Xiaomin Wang, Gang Hu, Jiawei Zhou

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A B S T R A C T

Parkinson’s disease (PD) is one of the most devastating neurodegenerative disorders. The underlying mechanisms of the characteristic neurodegeneration in the substantia nigra (SN) are still not fully understood. To better understand the molecular events occurring in the SN of PD brain, we used the culture-derived isotope tag–based quantitative proteomics to compare the protein expression profiles in the nigral tissue of PD patients and control subjects. We identified a total of 11 differentially expressed proteins, including alphaB-crystallin (Cryab). Both the levels and pattern of Cryab expression in the SN were validated. It was revealed that Cryab was markedly upregulated in the SN of PD brain. Cryab expression was also upregulated in reactive astrocytes and microglia in a neurotoxin-induced mouse PD model. Moreover, we showed increased expression of Cryab in cytoplasmic inclusions in a subset of glial cells in Parkinsonian brain. Thus, we identified Cryab that is highly expressed in the SN of PD brain and may be involved in the glial pathology during dopaminergic neuron degeneration in PD.

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1. Introduction

Parkinson’s disease (PD) is a common neurodegenerative disorder affecting millions of people around the world. It is characterized by selective degeneration of dopaminergic (DA) neurons in the substantia nigra (SN). Patients with PD are afflicted by severe motor symptoms including resting tremor, bradykinesia, rigidity, and postural instability. After decades of intensive investigation, several hypotheses regarding the causes and pathogenesis of PD have been proposed, such as oxidative stress, mitochondrial dysfunction, protein aggregation, and inflammation (Foltynie and Kahan, 2013; Jenner, 1991; McGeer et al., 2001; McNaught and Olanow, 2006; Mizuno et al., 1998). However, no curative therapeutic approach for PD has been successfully developed until now based on the current understanding of the disease, highlighting the importance of identifying new therapeutic targets.

The application of high-throughput approaches, such as DNA microarray and proteomics, has dramatically enhanced our understanding of the pathophysiology of several neurologic diseases including PD. DNA microarray analysis evaluates gene expression on a genome scale by using brain tissues from animal models or human patients. By using this powerful method, a wide variety of genes, including PGC-1α, that is linked to pathogenic changes in PD has been identified (Mandel et al., 2005; Simunovic et al., 2009; Zheng et al., 2010). However, given the discrepancy between the messenger RNA and protein abundance in the biological samples, proteomics has the advantage of allowing direct comparison of the changes in protein levels among various conditions.

One of the drawbacks of traditional proteomic approaches is the inability to make quantitative comparison on the absolute or relative protein levels across biological samples. Recent advances in the development of quantitative methods in proteomics provide unprecedented opportunity to quantify protein levels in complex biological samples. One of such methods is based on the use of culture-derived isotope tags (CDITs), in which the isotopically labeled proteins from cell culture were introduced as internal standards to quantify the protein levels in complex tissue samples.
Here, we quantitatively compared the proteomes of the SN from PD patients and healthy controls by using CDIT method. We found 11 proteins that displayed >1.5-fold change between control and disease groups. Among the 11 proteins, the upregulation of alphaB-crystallin (Cryab) was validated by using western blot in human brain samples. Immunohistochemistry revealed aberrant expression patterns of Cryab in the SN of PD patients and neurotoxin-induced rodent PD model. Our study provides compelling evidence that Cryab is involved in PD pathogenesis.

2. Materials and methods

2.1. Human tissue collection

Fresh-frozen ventral mesencephalic tissues and tissue sections were obtained from The Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands. All the materials have been collected from donors for or from whom a written informed consent for a brain autopsy and the use of the material and clinical information for research purposes had been obtained by The Netherlands Brain Bank. For proteomic and post hoc biochemical analyses, frozen brain tissues from 3 pairs of age- and gender-matched PD patients and healthy controls (Supplementary Table S1) were used. After 3 washes by cold phosphate-buffered saline, brain tissues were homogenized in 50-mM Tris (pH 7.4), 150-mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, and 1% sodium deoxycholate. A set of paraffin-embedded sections from 4 PD and 3 healthy control subjects were obtained from the same source and were used for immunohistochemical analysis. All the PD subjects were clinically and neuropathologically diagnosed, and the healthy control subjects were devoid of any neurologic diseases.

2.2. Quantitative proteomic analysis

Quantitative proteomic analysis was carried out as described previously (Ishihama et al., 2005) with a few modifications. Briefly, human SH-SY5Y neuroblastoma cells were grown in Dulbecco-modified Eagle medium deficient in l-leucine (Sigma-Aldrich, St Louis, MO, USA) with 10% fetal bovine serum (Gibco, NY, USA), and 13C-labeled l-leucine (Bio-Rad, Hercules, CA, USA) was added to the culture. After 6 passages, proteins were extracted and mixed with human brain samples. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were recovered and analyzed by liquid chromatography in combination with tandem mass spectrometry (MS) (Thermo, San Jose, CA, USA). After protein identification by MS, leucine-containing peptides were extracted. Their m/z and scan number information were used to extract mass chromatograms and the peak areas of peptides, and manual confirmation was done to correct peak areas. Each peak was quantified relative to its corresponding isotopically labeled peak from SH-SY5Y cells, which were used as comprehensive internal standards to normalize the variations of sample preparation and analysis. Finally, the amount of each peak was compared in different tissue samples relative to SH-SY5Y cells.

2.3. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of PD

Adult male C57BL/6 mice obtained from Shanghai Laboratory Animal Center, Chinese Academy of Sciences, were maintained on a 12-hour light-dark cycle at 23 °C with food and water ad libitum. All the procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)—induced mouse model of PD was prepared as described previously (Jackson-Lewis and Przedborski, 2007). Briefly, the mice (8- to 10-week olds) received 4 injections (with 2-hour intervals) of MPTP (20 mg/kg, intraperitoneal; Sigma-Aldrich) or saline in 1 day. Seven days after the last injection, mice were anesthetized by pentobarbital sodium and perfused transcardially with saline, followed by 4% paraformaldehyde. Brains were collected and postfixed until use.

2.4. Western blot analysis

Western blotting was performed as described previously (Li et al., 2006). The primary antibodies used were mouse monoclonal antibody against β-actin (1:5000, Sigma-Aldrich) and monoclonal antibody against Cryab (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed and incubated with appropriate secondary antibodies (1:5000, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Membranes were visualized and digitized with ImageQuant (LAS-4000; Fujiﬁlm, Tokyo, Japan). Optical densities of bands were analyzed by using ImageReader software (Fujiﬁlm).

2.5. Immunohistochemistry

Immunostaining on paraffin-embedded sections of human midbrain were performed. The sections were treated to retrieve antigens followed by incubation with 0.3% H2O2 in phosphate-buffered saline for 30 minutes to bleach the endogenous peroxidase. Sections were incubated with primary mouse anti-Cryab monoclonal antibody (1:200, Santa Cruz Biotechnology) followed by biotinylated secondary antibodies (1:800; Jackson ImmunoResearch Laboratories). Immunosignals were visualized by using 3,3-diaminobenzidine (Sigma-Aldrich), and images were captured by a microscope (BX51; Olympus, Tokyo, Japan). For mouse brain sections (25 μm thickness), similar protocols were used. For double immunofluorescent staining, mouse brain sections were incubated with either polyclonal rabbit anti-glial fibrillary acidic protein (GFAP, 1:1000; Dako, Denmark) or ionized calcium-binding adapter molecule 1 (Iba1, 1:500; Wako, Wakayama, Japan) antibody followed by incubation with secondary antibody conjugated with Alexa488. The same sections were then incubated with monoclonal Cryab antibody (1:200; Santa Cruz Biotechnology), followed by incubation with secondary antibody conjugated with Alexa555. Images were captured and analyzed by using a laser confocal microscope (Nikon, Tokyo, Japan).

2.6. Statistical analysis

Statistical analysis was performed using statistical software (GraphPad Prism, version 4.0; GraphPad Software, Inc, La Jolla, CA, USA). The control group was compared with the PD group using Student t test. Differences were considered significant when p values were <0.05.

3. Results

To investigate the molecular events occurring in PD brain, we quantitatively compared the proteomes of the nigral tissue from the 3 patients with PD and 3 age- and gender-matched healthy control subjects, using the CDIT-based quantitative proteomic approach. The human DA SH-SY5Y cells were cultured in 13C-labeled leucine-rich medium and then mixed with human brain samples from either PD or healthy controls to serve as an internal standard. A total of 3934 proteins were identified by MS, among which 229 proteins
were presented in all the samples. To evaluate the coverage of the 229 proteins in terms of their cellular and functional distributions, we analyzed these proteins by NetAffx software (Liu et al., 2003). It was revealed that the cellular and functional distributions of these proteins were highly divergent, indicating good coverage of the mass spectrometry readouts (Supplementary Fig. S1A and B).

We then assigned proteins with >1.5-fold changes in all the 3 pairs of brain samples as differentially regulated proteins in PD. A total of 11 such proteins were identified, 6 of which, including Cryab, hyaluronan and proteoglycan link protein 2 (Hapln2), coiled-coil domain containing 94 (Ccd94), membrane-associated progesterone receptor component 1 (Pgrmc1), heterogeneous nuclear ribonucleoprotein U-like 2 (Hnrnpul2), and proteasome activator complex subunit 2 (Psme2), were upregulated in the SN of patients with PD compared with control subjects. The 5 proteins that were downregulated in PD samples were DUT protein (Dut), potassium channel tetramerization domain-containing protein 13 (Kctd13), tubulin alpha-1 chain (Tba1), ruvB-like AAA ATPase 1 (Ruvb1), and annexin a1 (Anxa1) (Supplementary Table S2). Considering that the samples used to generate this data set were from patients at late-stage PD when >50%–70% of DA neurons had already degenerated, the downregulation of some of these proteins may partially result from DA neuron loss.

To validate whether the expression levels of the differentially expressed proteins were truly altered, Cryab was selected for further analysis. It was shown that expression levels of Cryab protein were markedly increased in the nigral tissue of patients with PD compared with control subjects (Fig. 1A and B). Consistent with these data, MPTP administration in mice also elicited robust increases in Cryab expression. Immunohistochemical analysis revealed that in the SN of MPTP-treated mice, there was a dramatic increase of Cryab expression in the SN, as evidenced by the dramatically elevated intensity of Cryab immunosignals in the SN of MPTP mouse model of PD compared with the control (Supplementary Fig. S2A). Notably, aberrant Cryab expression occurred in reactive glial cells, indicating that Cryab-positive glial cells represent the classical glial scar in the SN of MPTP mouse PD model. Moreover, double immunofluorescent staining showed that Cryab was expressed in reactive astrocytes (Supplementary Fig. S2B), as manifested by colocalization of Cryab and GFAP in the SN of MPTP-treated mice. Interestingly, colocalization of Cryab and Iba1, a marker for microglial cells, was also observed in the SN of MPTP-treated mice (Supplementary Fig. S2C), whereas resting microglial cells in control subjects were devoid of Cryab immunosignals (data not shown). Taken together, these results suggest that Cryab is associated with gliosis in a mouse PD model. Elevated Cryab may be neuroprotective by preventing neuronal degeneration induced by pathogenic stimuli.

To determine the cellular basis of Cryab upregulation in human brain, we investigated the expression pattern of Cryab in the SN of an independent cohort of PD patients and healthy controls by using immunohistochemistry. These PD patients had been pathologically diagnosed by the presence of alpha-synuclein-positive intracytoplasmic inclusions (Lewy body), in addition to the clinical diagnosis. Indeed, the alpha-synuclein–positive intracytoplasmic inclusions were observed in the SN of all 4 subjects.

![Fig. 1. AlphaB-crystallin (Cryab) is upregulated in the substantia nigra (SN) of patients with Parkinson’s disease (PD). (A) Representative western blots showing Cryab expression in the SN of PD patients and healthy controls. (B) Quantitative data shown in (A). Data are expressed as mean ± standard error of the mean (n = 3). *p < 0.05. (C–K) Cryab expression is elevated and aggregated in glial cells in the SN of PD. Immunohistochemical staining for Cryab in the SN of the subjects with PD (G–K) or controls (C–F) are shown. (E) and (F) Enlarged images of the squared areas in (C) and (D), respectively. Cryab expression in most of the cells in the control subjects is relatively low. However, in some cells, the expression level of Cryab is higher, showing intense immunosignals in both the cell body and cell extensions (arrows). (H–I) Enlarged images of the squared areas shown in (G). In PD brain, Cryab expression is significantly elevated, and the protein aggregates in a fraction of glial cells in PD are Cryab positive (arrowheads). Scale bars: (C), (D), and (G), 50 μm; (E), (F), (H), and (I), 20 μm; and (J) and (K), 8 μm.](image-url)
with PD, but not in the 3 controls in our immunohistochemical staining (data not shown). Cryab immunohistochemical staining revealed that Cryab was primarily localized in glial cells (Fig. 1C and D). In the brain of healthy subjects, the expression of Cryab in most of the cells was relatively low, although the expression levels of glial Cryab varied from one cell to another, and some of the Cryab-positive cells displayed higher intensity of immunoreactivities (Fig. 1C–F). In contrast, in the PD brain, the immunosignals of Cryab appeared to be significantly elevated compared with the controls (Fig. 1G). Interestingly, the increased expression of Cryab was observed in intracytoplasmic inclusions in glial cells but not in neuronal cells. Cryab was also observed in the neuropil thread pathology in cellular processes (Fig. 1H–K). In a few cases, cells with Cryab-positive inclusion were dismantled (Fig. 1K), suggesting that they were dying cells during neurodegeneration.

4. Discussion

In the present study, we investigated the proteome profiles of PD by applying CDIT-based quantitative proteomics approach. Eleven differentially expressed proteins were identified in the SN of PD, setting the basis for further investigation. The dysregulated proteins in PD are associated mainly with cell survival, protein degradation, and neuroinflammation, supporting leading hypothesis on PD pathogenesis.

CDIT-based quantitative proteomics uses isotope-labeled proteins from cell culture as internal standards to quantify the same proteins in the complex tissue samples. In the practice, usually 1 or 2 cell lines are used to generate isotope-labeled proteins that are unlikely to cover all the proteins expressed in tissue samples. Therefore, only those proteins that are expressed in both the cell line(s) and tissues have a good chance of getting quantitated. Despite the limitations, this approach is still considered very powerful, because of its quantitative nature, comparing with traditional proteomic approaches that generate descriptive data that are difficult to validate.

Among the upregulated proteins identified by our proteomic analysis, HaplN2 had the highest fold change. This protein is known to be localized in the extracellular matrix of the white matter in the adult central nervous system (CNS) and predominantly expressed by neurons (Hirakawa et al., 2000; Oohashi et al., 2002), functioning in the diffusion barrier formation and conduction velocity in the CNS (Bekku et al., 2010). However, the exact roles of HaplN2 in DA neurons and how it involves in PD pathogenesis are totally unknown. Pgrmc1 is a membrane-associated component of progesterone receptor and expressed by neurons, astrocyte, and microglia. Interestingly, Pgrmc1 is upregulated in neurons and astrocyte after traumatic brain injury and in microglia when stimulated by lipo-poly saccharide or other inflammatory insults (Bali et al., 2013a, 2013b; Meffre et al., 2005). Psme2 is one of the key regulators in the proteasome activator PA28 complex. The upregulation of Psme2 in PD brain may reflect the cellular responses to aggregated proteins such as alpha-synuclein, which is a key player in the pathogenesis of PD in both the familiar and sporadic cases. Importantly, Psme2 is also involved in the cellular immune responses by regulating the processing of major histocompatibility complex class I molecules (Kloetzel et al., 1999; Sijts et al., 2002).

We found that 5 proteins, including Anxa1, Dut, Kctd13, Tuba4a, and Ruvb1, were downregulated in the brain of patients with PD. Previous comparative study of annexins in human brain found intense cytoplasmic Anxa1 immunoreactivity in neurons, although it may also expressed in reactive astrocytes and microglia (Eberhard et al., 1994; McArthur et al., 2010). Interestingly, some of the downregulated proteins in PD are important regulators for cell survival. Dut is an enzyme for deoxyuridine triphosphate hydrolysis and a key regulator of intracellular deoxyuridine triphosphate pools in the cell. Mitochondrial Dut is constitutively expressed and can prevent apoptotic cell death induced by exogenous and endogenous stresses (Williams et al., 2011). Ruvb1 is an ATPase with diverse biological functions including, together with Ruvb2, chromatin remodeling, transcription regulation, mitosis, and assembly of telomere complex (Bauer et al., 2000; Ducat et al., 2008; Jonsson et al., 2001; Venteicher et al., 2008; Wood et al., 2000). Further studies are required to investigate the precise function of these differentially expressed proteins in PD pathogenesis.

Of the differentially expressed proteins identified in the SN in this study, Cryab, which is expressed mainly in astrocytes and oligodendrocytes in the adult brain, is known to be associated with a variety of pathologic conditions. Cryab is a member of small heat-shock protein family that actively responds to various cellular stresses such as aberrant protein aggregation under pathologic conditions and confers enhanced stress resistance on cells. Previous studies showed that Cryab was involved in multiple neurologic disorders such as neurodegenerative diseases such as parkinsonism, Alexander’s disease, amyotrophic lateral sclerosis, PD, PD dementia, and prion disorders (Braak et al., 2001; Dabir et al., 2004; Head et al., 1993; Iwaki et al., 1989, 1992; Renkawek et al., 1999; Shinozawa et al., 1993; Wang et al., 2013). These observations strongly suggest that Cryab plays an important role in the pathogenesis of age-related CNS diseases. They also support the notion that PD share common pathogenic mechanism(s) to some extent with other neurodegenerative diseases and these diseases could potentially be targeted by similar therapeutic strategies. Interestingly, the expression pattern of Cryab in PD revealed in the present study is distinct from those reported by others (Braak et al., 2001; Renkawek et al., 1999). In their studies, few of GFAP+ astrocytes reacted with Cryab antiserum were seen (Braak et al., 2001; Renkawek et al., 1999), despite that Cryab-positive neurons were evidenced in the cerebral cortex, amygdala, and hippocampus in PD brain (Braak et al., 2001). Moreover, Cryab-positive astrocytes can only be identified in PD dementia, a type of dementia that develops in a patient who is originally diagnosed with Parkinson’s, but not in those with typical PD (Renkawek et al., 1999), suggesting a strong correlation between aberrant Cryab expression and cognitive impairments in these patients (Renkawek et al., 1999). In contrast, in the present study, our investigation on Cryab expression in the SN of patients with PD using proteomic approach in combination with classic biochemistry and histology provided compelling evidence that Cryab was elevated in glial cells, but not in neuronal cells, in the SN of patients with PD. The discrepancy in Cryab expression pattern between our observations and those described by others may be attributable to the different Cryab antiserum used.

One of the remarkable features of the gliosis in PD presented here is the formation of abnormal Cryab-immunoreactive cytoplasmic aggregation in glial cells in the SN of PD brain, which appears to be similar in morphology to the glial inclusions of both sporadic and familial tauopathies (Dabir et al., 2004). The impact of glial inclusions on glial cell function is not yet clear. We speculate that in the early stage of PD, Cryab may be upregulated in reactive glial cells in response to inflammatory insults, such as MPTP as shown in Supplementary Fig. S2, and functions as a neuroprotective molecule, given that Cryab is known as a key negative regulator of neuroinflammation (Masl onion et al., 2005a, 2005b; Shao et al., 2013). As the disease progresses, detrimental factors induce the formation of glial inclusions via the sequestration of cytoplasmic proteins including Cryab through a yet-unidentified mode of action, thus tip the balance of immune homeostasis toward neuroinflammation. Indeed, recent work in our laboratory has shown that dramatic reduction in the levels of free Cryab protein in astrocytes, induced by downregulation of astrocytic dopamine D2...
receptor that occurs in aging brain and PD, significantly promotes proinflammatory gene expression (Shao et al., 2013). The aggregation of Cryab causes the impairment in Cryab-mediated protein handling and clearance promoting the recruitment of other proteins into aggregates in reactive glial cells and ultimately contributes to the dysfunction of glial cells (Fig. I and K) and neurodegeneration.

In conclusion, the present study showed that Cryab was markedly upregulated in the SN of PD patients where Cryab was present in glial cell inclusions. Further research is needed to determine whether Cryab-containing inclusion is formed in early stage of PD and exactly how Cryab is sequestered into the glial inclusion. It is of interest to note that the relevance between most of the differentially expressed proteins identified in our proteomic analysis is largely unexplored. Future studies are also required to further study the potential roles of these proteins in PD pathogenesis. The information generated from these studies will not only promote our understanding to the PD pathogenesis but also provide new insights into pathogenesis of neurodegenerative disorders as a whole.

Disclosure statement

The authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2015.01.015.

References

Bali, N., Morgan, T.E., Finch, C.E., 2013b. Pgrmc1: new roles in the microglial handling and clearance promoting the recruitment of other proteins into aggregates in reactive glial cells and ultimately contributes to the dysfunction of glial cells (Fig. I and K) and neurodegeneration.

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References

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coincidental with the astrogliosis in the brains of scrapie-infected hamsters and
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Med. 2, 52ra73.
Supplementary Fig. S1. Quantitative proteomic analysis of the substantia nigra of PD patients and healthy controls. (A, B) The subcellular (A) and functional distributions (B) of the 229 proteins which were identified by mass spectrometry and presented in all the samples.
**Supplementary Fig. S2.** Upregulation of Cryab in reactive astrocytes and microglia in the SN of MPTP mouse model of PD. (A) Immunohistochemical staining for Cryab in the ventral mesencephalon of adult male mice administered with either saline or MPTP. Arrows indicate the SN pars compacta (SNpc). Cryab is up-regulated in reactive glial cells in the SN of MPTP-treated mice. Cryab-positive glial cells represent the classical glial scar in the SNpc (arrows) of MPTP-treated mice. (B, C) Upregulation of Cryab in reactive astrocytes (B) and microglia (C). Arrows indicate double-labeled cells. Scale bars, A, 100 μm; B and C, 20 μm.
**Supplementary Table S1.** Summary of the demographic and clinicopathological data on the three Parkinson’s disease and three control cases used in this study

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<td>5 hrs and 10 min</td>
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**Supplementary Table S2.** List of differential expressed proteins that showed more than 1.5-fold changes across all three matched pairs of samples.

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<td>IPI00218918</td>
<td>0.25 ± 0.16</td>
</tr>
</tbody>
</table>

* denotes the average of fold-changes of a specific protein in the three pairs of PD and control subjects which were used to generate the proteomic data (mean ± SEM, n=3).