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Residual substantia nigra neuromelanin in Parkinson’s disease is cross-linked to α-synuclein

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Abstract

The pigmentation of substantia nigra pars compacta dopaminergic neurons is due to the presence of neuromelanin, an irregular macromolecular pigment belonging to the family of melanins. Depletion of neuromelanin in Parkinson’s disease is typically indicated by loss of brown color in this area. Unlike that from controls, the pigment extracted from substantia nigra of parkinsonian patients seems to be mainly composed by highly cross-linked, protease-resistant proteic material and the neuromelanin macromolecule appears to be a minor presence. In the present paper we describe the isolation by SDS-PAGE of this proteic component after cleavage of the melanin backbone under solubilizing conditions. A single band is observed, which has been identified as α-synuclein by western blotting. As expected, the same process performed on a control specimen did not show occurrence of any major proteic component. Nevertheless, extraction from a 91 years old control with Lewy bodies displayed minor α-synuclein immunoreactive aggregates, whereas inclusion of free α-synuclein was not observed at all. Results reported here support the view that α-synuclein accumulates within substantia nigra neurons and is entrapped in pigment granules during neuromelanin biosynthesis, i.e. before the melanin depletion characteristic of Parkinson’s disease starts.

Keywords: α-synuclein; Lewy bodies; Neuromelanin; Parkinson’s disease; Western blotting

1. Introduction

Neuromelanin is the pigment belonging to the melanin family responsible for the dark color of a region of the midbrain called substantia nigra pars compacta (SNpc) (Prota, 1992; Aime et al., 1994, 2000). This area of the human brain undergoes severe degeneration during the development of Parkinson’s disease (PD), the most common neurodegenerative disorder after Alzheimer’s disease (AD), with a prevalence of 2–3% among people over 65 years (Lang and Lozano, 1999). Typical motor symptoms are related to depletion of dopaminergic neuromelanin-containing neurons in SNpc with subsequent loss of dopaminergic efferents to the striatum (Lang and Lozano, 1999). Histological evidence shows that the more pigmented neurons are the first ones being degenerated. The pathogenesis of PD is not known, although there are suggestions that reactive oxygen species (ROS), by-products of the oxidative metabolic pathway, may result in the damage of cell membranes through addition of unsaturated bonds in the lipid bilayer. Oxidant stress conditions may, in turn, be strictly connected to excitotoxicity, or to mitochondrial dysfunction (Olanow and Tatton, 1999; Pocernich et al., 2001; Orth and Schapira, 2002). The SN is a preferential candidate to oxidative damage, since it contains oxidizable dopamine, neuromelanin, polyunsaturated fatty acids, iron, and relatively low antioxidant complement (Olanow and Tatton, 1999). Normally, ROS are buffered by a number of enzymatic control systems present both in the cell and in the extracellular environment. The increased turnover of ROS is usually due either to a failure in the scavenging mechanisms, or to an increase in their production (Halliwell and Gutteridge, 1989).

From the neuropathological viewpoint, PD is characterized by eosinophilic cytoplasmic inclusions, filamentous Lewy bodies (LB), in dopaminergic neurons of SNpc and of...
other pigmented nuclei. LB are composed by several interacting proteins, including α-synuclein, and are extensively ubiquitylated (Borden, 1998; Spillantini et al., 1998; Chung et al., 2001; Shimura et al., 2001). α-Synuclein is a small (14 kDa) acidic presynaptic protein constituted of 140 aminoacids organized in three domains: the N-terminal amphibatic repeat region, the non-Aβ amyloid component (NAC) region, and the C-terminal acidic region (Borden, 1998). The NAC peptide has been isolated as a secondary component of the extracellular plaque in AD patients, and it was supposed to be the responsible for a seeding process that initializes the plaque deposition by Aβ peptides. In recent years, two missense mutations in the α-synuclein gene (A53T, A30P) have been linked to rare early-onset familiar forms of PD (Mizuno et al., 2001). Recently, the effects of changes in the α-synuclein sequence on the enhanced susceptibility of cells to ROS have been reported (Kanda et al., 2000; Tabrizi et al., 2000), providing a pathogenic link between α-synuclein aberrations and a putative role of ROS in the cell death mechanism in Parkinson’s disease. At high concentration wild-type and mutant α-synuclein form non-fibrillar oligomers which assembly into fibrils with an increase of beta-sheet character; it has been demonstrated that both PD mutations accelerate the oligomerization enhancing the aggregation process observed in the wild-type protein (Narihi et al., 1999). An analysis of the water dynamics in ex vivo specimens of SNpc has shown a differential deposition of cytosolic proteins with respect to age-matched controls (Lopiano et al., 2000b). In a recent paper, we observed that the organic component of PD neuromelanin (but not that one from control patients) is mainly composed by protease-resistant proteic material (Aime et al., 2000). Although the experimental approach used at that time (i.e. solid-state NMR spectroscopy) was not addressed at the assignment of the proteic component, the melanoprotein specimen has been characterized in terms of its iron binding (and consequently pro-oxidant) capacity (Lepzano et al., 2000a). In the present paper, we describe the detection of immunoreactive α-synuclein in melanoprotein isolated from SNpc of patients affected by PD after cleavage of the melanin backbone under solubilizing conditions (Aime et al., 1991). In a similar way, different α-synuclein immunoreactive components have been observed in neuromelanin of a control patient with Lewy bodies, whereas no immunoreactivity was observed in other controls.

2. Experimental procedures

Specimens of human SN from two patients having known PD history were provided by The Netherland’s Brain Bank (The Netherland’s Brain Bank, Amsterdam. Each specimen was accompanied by histopathological report, showing deposition of pigmented dopaminergic neurons and the occurrence of extracellular neuromelanin granules as well as Lewy bodies in surviving neurons. Three control specimens have been obtained from the same brain bank with a similar histopathological report. One of the control specimens, from a patient died at the age of 91 years, showed the occurrence of Lewy bodies probably related to aging. In this case, extensive depigmentation of SNpc and extracellular neuromelanin granules characteristic of PD were not observed. The extraction of neuromelanin was carried out as reported elsewhere (Aime et al., 1994, 2000). The residual pigment was collected by centrifugation, washed several times with saline and reacted with 0.2 M aqueous ammonia containing 1% H2O2 for 2 h at 25°C, in order to break down the melanin framework as reported for specimens from different origin (Aime et al., 1991). The reaction suspension was centrifuged and an aliquot of the supernatant was mixed with an equal volume of the protein denaturation buffer according to standard SDS-PAGE procedure and separated on a vertical 15% polyacrylamide gel.

Western blotting was performed by electrotransfer on a Bio-Rad semidry transfer cell (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer’s instructions. Monoclonal anti-α-synuclein antibody was obtained by Zymed Laboratories Inc. (San Francisco, CA, USA). Detection of the immunoreactive band was achieved by enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden), with an anti-mouse-IgG antibody linked to horseradish peroxidase (Sigma Chem. Co., St. Louis, MO, USA). All results were obtained as duplicates on separate specimens, with the exception of the control with Lewy bodies that was available as a single specimen.

3. Results

Fig. 1A resumes the SDS-PAGE analysis of the proteic component of isolated neuromelanin in SN specimens from a control patient, two PD patients and a control patient (91 years old) with Lewy bodies, respectively, after transfer on nitrocellulose membrane and staining with Ponceau red. While Ponceau red staining does not show any proteic component in the control specimen within the sensitivity range, the oxidative breakout of the melanin framework in the PD specimen yields one major band (approximate MW about 18 kDa). According to the low sensitivity of this dye, we cannot exclude the presence of other protein components in the mixture; on the other hand, the marked stain observed for this band allows us to assign it to the main proteic component of the PD melanoprotein, which was observed by means of solid-state NMR spectroscopy (Aime et al., 2000). Previously reported NMR spectra of isolated NM specimens from controls did not show the occurrence of a major protein component, in agreement with the absence of intense stain observed here in the control lane.

When the neuromelanin extracted from the SNpc specimen of the control with Lewy bodies is considered, a more complicated proteic presence is observed. In spite of the occurrence of Lewy bodies, the sample showed regular...
pigmentation, and an amount of neuromelanin was extracted that compared to that of other control specimens. However, the melanin cleavage procedure revealed the occurrence of at least five proteic components.

In order to search for the occurrence of α-synuclein (or related oxidation products) in the oxidation mixture, we probed the blot with a monoclonal anti-α-synuclein antibody (Fig. 1B). Western blotting confirmed the absence of α-synuclein released by breakdown of the control NM specimen, whereas the main protein component observed in PD specimens appears to be immunoreactive against this antibody, and no other peptide or protein is detected beyond sensitivity. Furthermore, neither fragments, nor aggregates have been observed here, since this is the only band that reacts with the anti-α-synuclein antibody. It appears thus that the main component of protease-resistant proteinaceous material co-extracted with residual neuromelanin from SNpc of patients with PD history has dimension and immunoreactivity identical to those of α-synuclein (although the molecular mass is 14 kDa, α-synuclein has an irregular electrophoretic mobility that leads to an apparent molecular mass of 17–18 kDa). Since the solubilization process is aimed at breaking the melanin component, entire α-synuclein should be released during the oxidative attack. The extensive proteolytic digestion that the specimen is undergoing at the end of the melanin extraction procedure does not have any effect on this component. Rather, it should be noticed that a prolonged exposition to the cleavage reactant would give rise to extensive protein degradation with consequent smearing of the band (not shown). On the other hand, western blotting of the right-hand lane (control with LB) shows anti-α-synuclein immunoreactivity in the three components at higher molecular weights out of the five observed in Fig. 1A. It is worth to note that none of these bands corresponds to the apparent molecular weight of about 18 kDa usually observed for α-synuclein (lanes PD in Fig. 1).

4. Discussion

Melanin biosynthesis involves a number of radicalic intermediates (mainly semiquinones) that can interact with amino acid sidechains of soluble proteins (Prota, 1992). As recently reported, spontaneous or enzyme-controlled oxidation of dopamine in the presence of serum albumin gave rise to a melanoprotein whose NMR spectrum indicated the cross-linking of the protein to the growing melanin structure. Even after extensive proteolytic treatment, characteristic protein signals are still present in the NMR spectrum (Aime et al., 2000). Conversely, we did not observe extensive cross-linking of proteins among several NM specimens extracted from control patients (Aime et al., 1994; Lopiano et al., 2000a). Therefore, α-synuclein should be present in reasonably large amounts in the cytosol of SNpc neurons of PD patients before the neuromelanin depletion starts. Actually, radicalic addition to organized structures like Lewy bodies or their precursors would give rise to the inclusion of LB components other than α-synuclein.

Concerning cleavage of neuromelanin from the elder with LB, we have to take into account the higher pigmentation of the sample, i.e. a much larger amount of neuromelanin was extracted, digested and loaded on the gel starting from the same amount of fresh tissue. Therefore, protein expression and immunoreactivity cannot be directly compared to that of the PD sample. Nevertheless, two aspects are worth of note: (a) a protease-resistant proteic component is co-extracted with neuromelanin; and (b) 18-kDa α-synuclein is not observed here, whereas three immunoreactive components are observed at higher molecular weights. Although it is difficult to draw a picture, at the present stage, of the inclusion of α-synuclein adducts in neuromelanin during aging, it seems remarkable that in either controls (lanes CO and LB) free α-synuclein is not entrapped within neuromelanin.

In conclusion, recovery of α-synuclein from residual neuromelanin isolated from PD specimens indicates that accumulation of the protein likely occurs in the early stages of the disease, i.e. before the involved neurons start to degenerate. Proteasomal impairment recently reported in PD
(McNaught and Jenner, 2001) could lead to higher levels of α-synuclein, which does not need to be ubiquitylated in order to be processed by the catalytic subunits of the proteasome (Tofaris et al., 2001). In addition, the absence of higher molecular weight α-synuclein isoforms (glycosylated and/or ubiquitylated forms) is in keeping with the recently proposed picture for parkin function in the disposal of excess (fibrillar) α-synuclein (Chung et al., 2001; Giasson and Lee, 2001; Shimura et al., 2001).

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References


