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Research report

α-Synuclein-positive structures induced in leupeptin-infused rats

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Abstract

Abnormal accumulation of α-synuclein is regarded as a key pathological step in a wide range of neurodegenerative processes, not only in Parkinson's disease (PD) and dementia with Lewy bodies (DLB) but also in multiple-system atrophy (MSA). Nevertheless, the mechanism of α-synuclein accumulation remains unclear. Leupeptin, a protease inhibitor, has been known to cause various neuropathological changes in vivo resembling those of aging or neurodegenerative processes in the human brain, including the accumulation of neuronal processes and neuronal cytoskeletal abnormalities leading to neurofibrillary tangle (NFT)-like formations. In the present study, we administered leupeptin into the rat ventricle and found that α-synuclein-positive structures appeared widely in the neuronal tissue, mainly in neuronal processes of the fimbria and alveus. Immunoelectron microscopic study revealed that α-synuclein immunoreactivity was located in the swollen axons of the fimbria and alveus, especially in the dilated presynaptic terminals. In addition colocalization of α-synuclein with ubiquitin was rarely observed in confocal laser-scan image. This is the first report of experimentally induced in vivo accumulation of α-synuclein in non-transgenic rodent brain injected with a well-characterized protease inhibitor by an infusion pump. The present finding suggests that the local accumulation of α-synuclein might be induced by the impaired metabolism of α-synuclein, which are likely related to lysosomal or ubiquitin-independent proteasomal systems.

Theme: Disorders of the nervous system
Topic: Degenerative disease: Parkinson's

Keywords: α-Synuclein; Leupeptin; Protease inhibitor; Immunohistochemistry; Immunoelectron microscopy

1. Introduction

Lewy bodies (LB) and Lewy neurites are universally recognized as pathological hallmarks of Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Although LB can be observed microscopically with hematoxylin-eosin stain, an immunohistochemical method for detection of LB with either anti-ubiquitin or anti-α-synuclein antibody is recommended [1,6]. α-Synuclein has been proven to be one of the major components of LB in PD and DLB [9,26]; however, aggregation of α-synuclein is also demonstrated in the brains of Alzheimer's disease (AD) patients [17]. In addition, two recent cases of patients with diffuse neurofibrillary tangles disease with calcification (DNTC) have shown that neurons containing α-synuclein-positive structures are widely distributed, especially in the amygdala, hippocampus, and upper temporal gyrus [32]. Because the pure form of DLB, that is, DLB without or with very few neurofibrillary tangles or senile plaques is known [13], the process of Lewy body formation would not comprise a simple linkage of NFT formations. On the contrary, the frequent coexistence of NFT and LB in AD brains indicated that there may be a relationship between the mechanism of tau

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accumulation and that of α-synuclein accumulation in the AD and DNTC brains, although these mechanisms are so far unknown.

Leupeptin, a protease inhibitor, is known to cause (1) the accumulation of lipofuscin-like granules in the neuronal perikarya and (2) the degeneration of neurites [4,7,27]. Furthermore, we previously reported that the long-term infusion of leupeptin into the rat ventricle caused cytoskeletal changes that included the formation of abnormal bundles of paired helical filament-like filaments with 20 nm diameter and periodic constrictions at 40 nm intervals in the cortical neuron [28]. We reported that these changes had some morphological resemblance to neuropathological features of Alzheimer's brains.

In the present study, we found, by the administration of leupeptin to the rat brain, that α-synuclein-positive structures appeared in various portions of the brain in response to the degeneration caused by leupeptin. Immunoelectron microscopic studies revealed that α-synuclein-positive materials accumulated in the swollen axons, especially in dilated presynaptic terminals and in the neuronal cell bodies. These results revealed that a disturbance of protein degradation causes not only cytoskeletal abnormality, as we reported before, but also α-synuclein accumulation in the neurons. In this study, it suggests that impairment of protein degradation might be closely related with neurodegenerative diseases including AD that is characterized by cytoskeletal abnormality with accumulation of tau, amyloid β, and α-synuclein. It was reported that decreased activities of proteasome were observed in AD brain [12], and that in cultured cells protease-dysfunction induced neuronal degeneration [10]. Therefore, we would like to raise the hypothesis that disturbed protein-degradative activities are initial causative events leading to neurodegenerative disorders, even though the cause of dysfunction of protein degradation was not clarified. Recently, in the study of polyglutamine disease, one of neurodegeneration diseases, dysfunction of proteasome was found to be an initial event in the degenerative processes, and it might support our thought.

Previously accumulation of α-synuclein was reported in transgenic-model mouse [25,30], or rodents injected with MPTP or rotenone [3,5,16]. Both of chemical compounds were not well characterized in biochemical function on the mechanisms of cytotoxicity yet, even though oxidative stress mechanisms are thought to be mediators in either compound. This is the first report of experimentally induced in vivo accumulation of α-synuclein in non-transgenic rodent brain injected with leupeptin, a biochemically well-characterized protease inhibitor, by an infusion pump.

2. Materials and methods

Twenty 8-week-old Wistar rats, weighting about 300 g, were used for the present study. Leupeptin (Peptide Institute Inc., Osaka, Japan) solution, dissolved in phosphate-buffered saline (pH 7.4) at 25 mg/ml, was infused with an osmotic minipump (Model 2002; Alzet, California, USA). The outline of the operation was described elsewhere [27]. The pump was connected by means of a Silastic tube to an intracerebroventricular cannula and implanted subcutaneously in the neck. The cannula was implanted stereotaxically (0.8 mm posterior to the bregma, 1.2 mm lateral to the midline) into the right lateral ventricle. Five rats were implanted with pumps containing only phosphate-buffered saline (PBS) and served as controls.

2.1. Immunomicroscopy

Following infusion for 14 days, the rats were anesthetized and killed by perfusion through the left ventricle with 4% buffered paraformaldehyde solution.

For immunomicroscopic study, brains were dissected and fixed for 24 h in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). The brains, including the brain stem and the spinal cord, were cut coronally at 3 mm thickness. The slices were rinsed with PB, dehydrated through graded alcohol, and embedded in paraffin. Paraffin sections at 10 μm were made, deparaffinated, and prepared for immunostaining. Immunostaining was performed with monoclonal anti-α-synuclein antibody synuclein-1 (1:500 dilution) (Transduction Laboratories, Lexington, USA) as the primary antibody [20]. Sections were incubated overnight at 4 °C. For the immunohistochemical detection of the primary antibody, sections were incubated with a secondary antibody (1:100 biotinylated anti-mouse; Vector Laboratories, Burlingame, USA) for 2 h at room temperature. After rinsing in PB, the sections were then incubated in avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, USA) for 1 h at room temperature. After three subsequent washings in PB, sections were visualized by diaminobenzidine tetrahydrochloride.

2.2. Immunoelectron microscopy

For immunoelectron microscopic study, the rats were perfused with 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% picric acid in 0.1 M PB (pH 7.4). Brains were fixed for 24 h in 4% paraformaldehyde, 7% sucrose in PB. The leupeptin-treated rat brains were cut by vibratome to make 50-μm-thick coronal slices and there were washed in PB. Sections were first incubated overnight with the anti-α-synuclein antibody (1:500 dilution) at 4 °C, then incubated for 2 h with the secondary antibody (1:100 biotinylated anti-mouse; Vector Laboratories, Burlingame, USA), and treated with the avidin–biotin–peroxidase complex for 1 h at room temperature. After three subsequent washings in PB, sections were visualized by diaminobenzidine (DAB) tetrahydrochloride. For the post-fixation for electron microscopy, sections were incubated with 4% paraformaldehyde and 0.1% glutaraldehyde in PB