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Retrograde dopaminergic neuron degeneration following intrastriatal proteasome inhibition

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Abstract

Recent studies have suggested that defects in the ubiquitin–proteasome system (UPS) contribute to the etiopathogenetic mechanisms underlying dopaminergic neuronal degeneration in Parkinson's disease. The present study aims to study the effects of proteasome inhibition in the nerve terminals of nigrostriatal dopaminergic neurons in the substantia nigra pars compacta (SNpc). Following a unilaterally intrastriatal injection of lactacystin, a selective proteasome inhibitor, dopaminergic neurons in the ipsilateral SNpc progressively degenerated with alpha-synuclein-immunopositive intracytoplasmic inclusions. When lactacystin was administered at a high concentration, the striatum was simultaneously involved, and alpha-synuclein-immunopositive extracytoplasmic granules appeared extensively within the SN pars reticulata (SNpr). In addition, during the retrograde neuron degeneration in SN, the level of heme oxygenase-1 immunopositivity, an oxidative stress marker, was markedly increased in SNpc neurons. These results reveal that intrastriatal proteasome inhibition sufficiently induces retrograde dopaminergic neuronal degeneration with abundant accumulation of alpha-synuclein in the SN.

Keywords: Proteasome; Lactacystin; Rat; Substantia nigra; Oxidative stress

The ubiquitin–proteasome system (UPS) plays important roles in controlling intracellular levels of various short-lived proteins. Although it has been considered that proliferating cells but not postmitotic cells are differentially vulnerable to proteasomal inhibition [6], recent studies have suggested that inhibition of UPS is sufficient to induce the death of non-proliferating cells such as neurons [15,21,22]. Proteasome inhibition may induce abnormal accumulation of ubiquitinated, misfolded, aggregated, or oxidated proteins that should be removed from cells, finally resulting in cell death [11]. Recent studies have suggested the possibility that UPS failure contributes to mechanisms underlying neuronal death in neurodegenerative diseases, particularly Parkinson's disease (PD) [17]. Based on recent progress in genetics research on familial parkinsonism, it is hypothesized that UPS failure and subsequent proteolytic stress may contribute to the etiopathogenesis that underlies dopaminergic neuronal degeneration. Gene mutations identified as the cause of familial PD, such as alpha-synuclein, parkin, and ubiquitin C-terminal hydrolase L1 (UCH-L1), may be capable of interfering with normal protein degradation by UPS [17].

Currently, one unconfirmed concept on the pathogenetic mechanism underlying dopaminergic neuronal degeneration is that the degenerative processes may start at the nerve terminals of dopaminergic neurons [1,7,23]. From an etiopathologic perspective, this possibility is relevant. For example, the parkin protein, mutations of which have been identified as causes of autosomal recessive familial Parkinson's disease (PARK-II), is transported from the Golgi apparatus to nerve terminals and then to synaptic vesicles [13]. Similarly, alpha-synuclein, mutations of which have been identified to cause the autosomal dominant familial parkinsonism (PARK-I), is a presynaptic protein that may play a role in neurotransmitter release and synaptic plasticity [2,5,12]. Since both parkin

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and alpha-synuclein are proteins that function in nerve terminals, it is possible that the UPS failure caused by mutant proteins may be induced primarily in the nerve terminals of dopaminergic neurons, subsequently inducing toxic insults in the cell body. The functional damage caused by the UPS failure in nerve terminals may play a key role in the pathogenetic mechanisms underlying dopaminergic neuronal degeneration. Therefore, it is relevant to study the effects of the inhibition of UPS in the nerve terminals of nigrostriatal dopaminergic neurons. In the present study, we therefore, aim to examine the retrograde effects of an intrastriatal injection of lactacystin, a selective proteasome inhibitor, on cells in SN.

Adult male Sprague–Dawley rats ($n = 36$), weighing 250–300 g at the time of surgery, were used. The animals were housed in cages and kept in a temperature-controlled room ($23 \pm 1\,^{\circ}C$) under a 12-h light:12-h dark cycle. Food and tap water were freely accessible. The experimental protocols were approved by the Wakayama Medical University's Animal Care and Use Committee. During the surgical procedure, animals were anesthetized with ketamine (50–60 mg/kg body weight) and xylazine (10 mg/kg body weight). The animals were placed on a stereotaxic frame (Narishige, Japan), and a small bar hole was drilled in the skull. For the intrastriatal injection of a proteasome inhibitor, a 30-G stainless-steel cannula connected to a 10 μl Hamilton microsyringe was slowly inserted into the striatum unilaterally with the following stereotaxic coordinates of the target site: 0.5 mm anterior and 3.5 mm lateral to the bregma, and 5.5 mm below the dura [20]. Lactacystin (Peptide Institute, Osaka, Japan), a selective inhibitor of proteasome, was dissolved in physiological saline (10, 1 μg/2 μl). These concentrations of lactacystin were chosen, on the basis of a pilot study. Lactacystin at the dose of 0.2 μg/2 μl or less did not produce sufficient toxic effects on dopaminergic neurons in SN. In sham-operated rats, 2 μl of saline was injected into the striatum. The drug solution was injected at a rate of 1 μl/min, and the injection cannula was kept in situ for an additional 3 min to avoid spread of the solution along the pipette track. At various time intervals after the intrastriatal injections of lactacystin, the rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde solution, and the fixed brains were immediately removed. After an overnight postfixation period, the brains were incubated in 30% sucrose for 24–48 h, frozen rapidly and stored at $-80\,^{\circ}C$. Free-floating frozen 40 μm-thick coronal sections were prepared for immunohistochemical studies. Before immunohistochemistry, selected sections were stained with cresyl-violet. In immunohistochemisry, the sections were incubated for 20 min in PBS containing 0.25% Triton-X and 1% hydrogen peroxide. After preincubation in 5% blocking serum, the sections were incubated for 24–48 h at $4\,^{\circ}C$ with the following primary antibodies: a mouse monoclonal anti-tyrosine hydroxylase (TH) antibody (Chemicon, 1:10,000), a rabbit polyclonal anti-alpha-synuclein antibody (Santa Cruz, 1:500), and a mouse monoclonal anti-heme oxygenase-1 (HO-1) antibody (Takara, 1:1000). The sections were rinsed and incubated with appropriate secondary biotinylated antibodies. The avidin–biotine-peroxidase method was used (ABC Elite®, Vector Laboratories), and the sections were developed in a solution containing 0.03% diaminobenzidine. To detect apoptosis, the in situ end-labeling method (ApopTag® in situ apoptosis detection kit (Intergen)) was used. The approximate extent of the SN lesion was assessed by quantifying the average loss of substantia nigra pars compacta (SNpc) neurons on the injected side compared with that on the intact side at the midlevel of SN (5.3 mm posterior to the bregma) at which the oculomotor nerve root divides SN from VTA. The total number of TH-immunopositive or HO-1-immunopositive neurons in SNpc was counted on selected slides. All counts were performed by a single observer who was unaware of the treatment. All data are expressed as mean ± S.E. ($n = 4$–5 per group). Difference in the number of TH- or HO-1—immunopositive cells was subjected to one-way analysis of variance (ANOVA). The Bonferroni–Dunn test was used for post hoc comparisons. The level of statistical significance was set at $P < 0.05$.

Following the intrastriatal injection of lactacystin (10 μg/2 μl), an ipsiversive deviated posture with spontaneous circling was induced. At 2–3 days postlesion, this markedly ipsiversive deviated behavior was observed, presenting a dystonic deviation of the body axis to the side ipsilateral to the side of intrastriatal injection, but gradually subsided within 1 week. Although quantitative analysis of the circling behavior was not performed, the asymmetrical behavior became less severe by 7 days postlesion. In rats treated with the low dose of lactacystin (1 μg/2 μl), a transient asymmetrical behavior was observed at 2–3 days postlesion.

Following the intrastriatal injection of a high dose of lactacystin (10 μg/2 μl), the striatum was not free from the direct toxicity of lactacystin; a massive lesion was induced in the striatum, with an extensive loss of neurons, surrounded by prominent reactive gliosis (Fig. 1a and b). At the center of the lesion, small, necrotic cavitations were occasionally observed. In addition, in the brain tissue regions immediately adjacent to the track of the injection needle that were directly exposed to the high concentration of lactacystin, such as the cerebral cortex and subcortical white matter, nonspecific tissue damage was also observed around the needle track but only to a slight degree. On the other hand, in rats treated with a low dose of lactacystin (1 μg/2 μl), the striatum did not show any identifiable tissue damage, except for regions immediately around tracking of the injection needle (Fig. 1b and c).

The intrastriatal injection of lactacystin-induced a marked, progressive loss of TH-immunopositive neurons in the ipsilateral SN (Fig. 1e–h). Statistical analysis of the number of TH-immunopositive neurons in SN demonstrated significant effects for time and brain side ($P < 0.001$). There was a significant interaction of time and side. The decrease in the number of TH-immunopositive neurons in SN was statistically significant at 7 days postlesion ($P < 0.001$) (Fig. 1h).