Mitochondrial Complex I Inhibitor Rotenone Inhibits and Redistributes Vesicular Monoamine Transporter 2 via Nitration in Human Dopaminergic SH-SY5Y Cells

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ABSTRACT

Parkinson's disease is a progressive neurodegenerative disorder characterized by selective degeneration of nigrostriatal dopaminergic neurons. Long-term systemic mitochondrial complex I inhibition by rotenone induces selective degeneration of dopaminergic neurons in rats. We have reported dopamine redistribution from vesicles to the cytosol to play a crucial role in selective dopaminergic cell apoptosis. In the present study, we investigated how rotenone causes dopamine redistribution to the cytosol using an in vitro model of human dopaminergic SH-SY5Y cells. Rotenone stimulated nitration of the tyrosine residues of intracellular proteins. The inhibition of nitric-oxide synthase or reactive oxygen species decreased the amount of nitrotyrosine and attenuated rotenone-induced apoptosis. When we examined the intracellular localization of dopamine immunocytochemically using anti-dopamine/vesicular monoamine transporter 2 (VMAT2) antibodies and quantitatively using high-performance liquid chromatography, inhibiting nitration was found to suppress rotenone-induced dopamine redistribution from vesicles to the cytosol. We demonstrated rotenone to nitrate tyrosine residues of VMAT2 using an immunocytochemical method with anti-nitrotyrosine antibodies and biochemically with immunoprecipitation experiments. Rotenone inhibited the VMAT2 activity responsible for the uptake of dopamine into vesicles, and this inhibition was reversed by inhibiting nitration. Moreover, rotenone induced the accumulation of aggregate-like formations in the stained image of VMAT2, which was reversed by inhibiting nitration. Our findings demonstrate that nitration of the tyrosine residues of VMAT2 by rotenone leads to both functional inhibition and accumulation of aggregate-like formations of VMAT2 and consequently to the redistribution of dopamine to the cytosol and apoptosis of dopaminergic SH-SY5Y cells.

Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized by selective loss of dopaminergic neurons in the substantia nigra, decreased striatal dopamine levels, and the presence of cytoplasmic eosinophilic inclusions, termed Lewy bodies, which result in extrapyramidal motor dysfunction (Dawson and Dawson, 2003). The etiology of this nigral dopamine neuronal degeneration still is not fully understood, although genetic analyses, epidemiological studies, neuropathological investigations, and new experimental models of PD have provided important new insights into the pathogenesis of PD (Shastry, 2001). An established hallmark of PD is reduced activity of brain mitochondrial enzyme complex I (Schapira et al., 1990). Complex I inhibitors, such as 1-methyl-4-phenylpyridinium ion (MPP⁺) and rotenone, have been shown to cause apoptosis of nigral dopaminergic neurons and, thereby, parkinsonian dysfunction (Greenamyre et al., 2001). These observations suggest that a defect in mitochondrial complex I activity may contribute to the neurodegenerative process in PD. These dopaminergic neurotoxins enhance reactive oxygen species (ROS) and nitric oxide (NO) production and are responsible for the production of a toxic factor, peroxynitrite (Koppenol et al., 1992). One of the targets of nitration is tyrosine hydroxylase (TH) (Blanchard-Fillion et al., 2001). This nitration occurs with administration of the parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to mice and after exposure of PC12 cells to either peroxynitrite or MPP⁺ (Ara et al., 1998; Blanchard-Fillion et al., 2001). Nitration of one or more tyrosine residues of TH was temporally associated with loss of enzymatic activity. The tyrosine nitration-mediated loss of

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ABBREVIATIONS: PD, Parkinson's disease; DAT, dopamine transporter; MPP⁺, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NAC, N-acetylcysteine; PBS, phosphate-buffered saline; NOS, nitric-oxide synthase; ROS, reactive oxygen species; HPLC, high-performance liquid chromatography; VMAT2, vesicular monoamine transporter 2; GBR12935, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine; L-NAME, N-nitro-L-arginine methyl ester hydrochloride; TH, tyrosine hydroxylase; 7-NI, 7-nitroindazole; SLO, streptolysin O; KG, potassium glutamate.

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TH activity parallels the decrease in dopamine levels, but this dopamine decrease alone is not sufficient to induce apoptosis because reserpine-induced depletion of intracellular dopamine does not lead to apoptosis (Watabe and Nakaki, 2007). Therefore, much less is known about the involvement of peroxynitrite in apoptosis induced by these toxins.

Dopamine is a neurotransmitter under physiological conditions. However, accumulating evidence indicates that dopamine may also serve as a neurotoxin and thereby participate in the neurodegenerative process. The mechanism of dopamine neurotoxicity is strongly linked to oxidative metabolism. Dopamine can be oxidized spontaneously in vitro or through an enzyme-catalyzed reaction in vivo to form ROS, free radicals and quinones. These oxidation products can damage cellular components such as lipids, proteins, and DNA (Halliwell, 1992). Because vesicular dopamine is protected from oxidative damage, dopamine may be expelled from vesicles in the neurodegenerative process, thereby becoming vulnerable to ROS and enzymatic metabolism. In fact, we reported that rotenone triggers dopamine redistribution from vesicles to the cytosol (Watabe and Nakaki, 2007). However, the mechanisms underlying dopamine redistribution have remained elusive.

Vesicular monoamine transporters (VMATs) translocate monoamines (serotonin, dopamine, norepinephrine, and histamine) from the cytoplasm into synaptic vesicles of monoaminergic neurons, neuroendocrine cells, and platelets. Transport is driven by an electrochemical proton gradient across the vesicular membrane, which is generated by a vacuolar H^+-ATPase (Kanner and Schuldiner, 1987). In mammals, two closely related isoforms of the monoamine transporter, termed VMAT1 and VMAT2, respectively, were identified (Erickson et al., 1992; Liu et al., 1992). These VMATs have 12 transmembrane domains and show a range of differences in their physiological and pharmacological properties. In addition to these differences, VMAT1 and VMAT2 differ in their tissue distributions. In particular, the transporter expressed in neural cells is VMAT2, not VMAT1 (Peter et al., 1995). VMAT2 is responsible for packing dopamine and other monoamines into vesicles in monoamine-containing neurons in nerve cell bodies and dendrites as well as into synaptic vesicles at nerve terminals (Nirenberg et al., 1996). Furthermore, VMAT2 provides neuroprotection by sequestering toxic substrates such as MPP^+ into vesicles (Liu et al., 1992). Previous studies have shown reduced expression of VMAT2 in nigral neurons of patients with PD (Harrington et al., 1996; Miller et al., 1999), suggesting a reduction in the neuroprotective effect provided by VMAT2 and consequent progression of neurodegeneration.

Human SH-SY5Y cells, subcloned from the SK-N-SH cell line demonstrated to express DA receptors and dopamine transporters, are often used as a dopaminergic neuron model. Indeed, rotenone induced apoptosis in SH-SY5Y cells (Watabe and Nakaki, 2004). The present study was undertaken to directly assess whether rotenone-induced nitration is involved in the redistribution of dopamine, which is then expelled from vesicles, and, if so, whether rotenone-induced nitration affects VMAT2 activity in human dopaminergic SH-SY5Y cells.

Materials and Methods

Materials. Rotenone, dopamine, GBR12935, *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME), 7-nitroindazole (7-NI), streptolysin O (SLO), thioflavin S, and anti-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO). Anti-mouse monoclonal dopamine antibody was obtained from AbCam Limited (Cambridge, UK) and anti-mouse monoclonal nitrotyrosine antibody (clone 1A6) from Millipore (Billerica, MA). Anti-rabbit vesicular monoamine transporter 2 (VMAT2) antibody was obtained from Millipore Bioscience Research Reagents (Temecula, CA). This antibody recognizes a major 80-kDa band, which represents fully processed VMAT2, and two minor bands at 62 and 40 kDa, which are also VMAT2 and presumably represent incompletely processed/glycosylated VMAT2 (Watabe and Nakaki, 2007). In the present study, a major 80-kDa band of VMAT2 alone was identified.

Cell Culture. SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C under 5% CO_2 in air.

Immunofluorescence Microscopy. Immunocytochemical studies were performed as described previously (Watabe et al., 1997, 2000). Cells were washed with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde for 20 min. Cells were permeabilized with PBS containing 0.2% Triton X-100 for 5 min and then washed three times with PBS. Incubation with primary antibody was carried out for 1 h at room temperature. Excess antibody was washed out three times with PBS. This was followed by incubation with an appropriate fluorophore-labeled secondary antibody for 1 h at room temperature in an area protected from light. After washing out the excess antibody three times with PBS, mounting was performed using a ProLong Antifade Kit (Invitrogen, Carlsbad, CA). For thioflavin S staining as described by Trimmer et al. (2004), fixed cells were incubated with 0.05% thioflavin S for 5 min and washed three times with 80% ethanol before the antibody incubations. Images were obtained by fluorescence microscopy (Axio Imager M1; Zeiss, Oberkochen, Germany).

Quantification of DNA Fragmentation. As described previously (Watabe et al., 2004), DNA fragmentation was measured using a Cell Death Detection ELISA^{PLUS} kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. This assay is useful for differentiating apoptosis from necrosis and can detect internucleosomal degradation of genomic DNA, which is a hallmark of apoptosis, by quantitatively measuring histone-associated DNA fragments.

Measurement of Dopamine. Dopamine was measured as described previously (Watabe and Nakaki, 2007). In brief, to measure dopamine amounts in the vesicle and cytosol fractions, the cells were treated with rotenone in the presence of 0.5 μM GBR12935 to prevent the release from the inside to the outside of the cell via dopamine transporters (DAT). The vesicle and cytosol fractions were prepared using a Synaptic Vesicles Isolation Kit (Sigma-Aldrich) according to the manufacturer's instructions. The vesicle fraction was lysed in 0.01 M hydrochloric acid, and trichloroacetic acid was added to the cytosol fraction (final concentration, 5%, w/v). After centrifugation (15,000g for 10 min at 4°C), the supernatant was immediately eluted at a flow rate of 0.5 ml/min and a potential of 750 mV using an EIKOMPAK SC-5ODS column (Eicom Corporation, Kyoto, Japan) as the HPLC system. The mobile phase consisted of 0.1 M citric acid/0.1 M sodium acetate, pH 3.5, 5 mg/ml EDTA, and 190 mg/l 1-octanesulfonic acid. The dopamine level in each sample was quantified by comparing the peak area with that of a dopamine standard solution.

Immunoblot Analysis. Immunoblotting was performed as described previously (Watabe et al., 1998). In brief, cells were lysed in a buffer containing SDS and mercaptoethanol, and the cell lysate was then boiled. Denatured proteins were separated on a polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (GE Healthcare UK Ltd., Chalfont St. Giles, Buckinghamshire, UK).

The membrane was incubated with a blocking solution [2% bovine serum albumin (Sigma-Aldrich) dissolved in PBS containing 0.2% Tween 20] for 1 h at room temperature, washed with PBS containing 0.2% Tween 20, and incubated with the first antibody dissolved in the blocking solution overnight at 4°C. After washing, the membrane was incubated for 1 h with horseradish-linked secondary antibody. Immunoreactive proteins were detected with an enhanced chemiluminescence system (GE Healthcare).

Immunoprecipitation. Immunoprecipitation was performed using a modification of the method described previously (Watabe et al., 2004). After treatment with rotenone, the cells were pelleted, washed twice in PBS, and lysed in lysis buffer (25 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail (Sigma-Aldrich). The cell lysates were then used for immunoprecipitation in the presence of an anti-nitrotyrosine antibody. Immune complexes were precipitated using Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and washed four times in lysis buffer. The precipitate was resuspended in buffer containing SDS and mercaptoethanol, boiled, and immunoblot analysis was then performed.

Dopamine Uptake. Dopamine uptake was measured essentially as reported previously (Höltje et al., 2000) but with minor modifications. In brief, the medium was removed, and cells were washed twice with PBS and once with potassium glutamate buffer (KG buffer) containing 150 mM potassium glutamate, 20 mM 1,4-piperazine diethanesulfonic acid, 4 mM EGTA, and 1 mM MgCl$_2$ adjusted to pH 7.0 with KOH, before being suspended in KG buffer. The cell suspension was mixed with SLO (1000 U/1 $\times$ 10^6 cells) dissolved in KG buffer containing 1 mM dithiothreitol and incubated for 10 min on ice. Unbound SLO was removed by centrifugation (1000g, 2 min, 4°C). The cell pellet was resuspended in KG buffer, distributed into individual tubes, and incubated for 10 min at 37°C to induce permeabilization and remove cytosolic components. Permeabilized cells were washed by adding 500 μl of ice-cold KG buffer and spun down for 2 min at 4°C. Uptake was initiated by adding 100 μl of KG buffer containing 2 mM Mg-ATP supplemented with 1 mM ascorbic acid and 1 mM dopamine. Incubation was performed for 10 min at 37°C and stopped by adding 1 ml of ice-cold KG buffer followed by a rapid centrifugation. After washing with ice-cold KG buffer, the cell pellet was lysed in 0.01 M hydrochloric acid. After centrifugation (15,000g for 10 min at 4°C), the supernatant was immediately injected into an HPLC system. Dopamine levels in each sample were quantified by comparing the peak area with that of a dopamine standard solution.

Statistical Analysis. Statistical significance was determined using either Student's t test for two group comparisons or analysis of variance with the Kruskal-Wallis test for multiple group comparisons. A p value of <0.05 was considered significant.

Results

In control cells, dopamine showed the same intracellular distribution as VMAT2, which is localized in vesicles (Fig. 1). With rotenone treatment, nuclei condensed and dopamine diffused throughout the cells, in contrast to the distribution of VMAT2 (Fig. 1). Thus, rotenone induced dopamine redistribution from vesicles to the cytosol in parallel with the apoptosis-inducing process. Rotenone is known to cause ROS generation during the process of apoptosis induction. It is also known that nitration of tyrosine residues on intracellular proteins is caused by peroxynitrite, which is formed by intracellular ROS and NO (Koppenol et al., 1992; Kuhn et al., 2004). To investigate the involvement of nitration in rotenone-induced neurotoxicity, we examined nitration of the tyrosine residues of intracellular proteins using anti-nitrotyrosine antibody. Rotenone increased the intracellular nitrotyrosine amount (Fig. 2A), and this increase was maintained in the late stage of rotenone-induced apoptosis (Fig. 2B). To confirm whether the peroxynitrite synthesized by ROS and NO caused the increase in nitrotyrosine, we examined the effect of L-NAME, an inhibitor of nitric-oxide synthase (NOS), or N-acetylcysteine (NAC), a scavenger of ROS on rotenone-induced nitration. Both L-NAME and NAC suppressed the rotenone-induced nitration of tyrosine residues on intracellular proteins (Fig. 2A), consistent with our previous report that NAC attenuated rotenone-induced DNA fragmentation in SH-SY5Y cells (Watabe and Nakaki, 2007). We also examined the effect of L-NAME on rotenone-induced apoptosis. L-NAME attenuated rotenone-induced DNA fragmentation in parallel with the degree of apoptosis (Fig. 2C). 7-NI, a specific nNOS inhibitor, yielded the same result as L-NAME (Fig. 2C). These results suggest that nitration of the tyrosine residues of intracellular proteins plays a role in rotenone-induced and apoptosis.
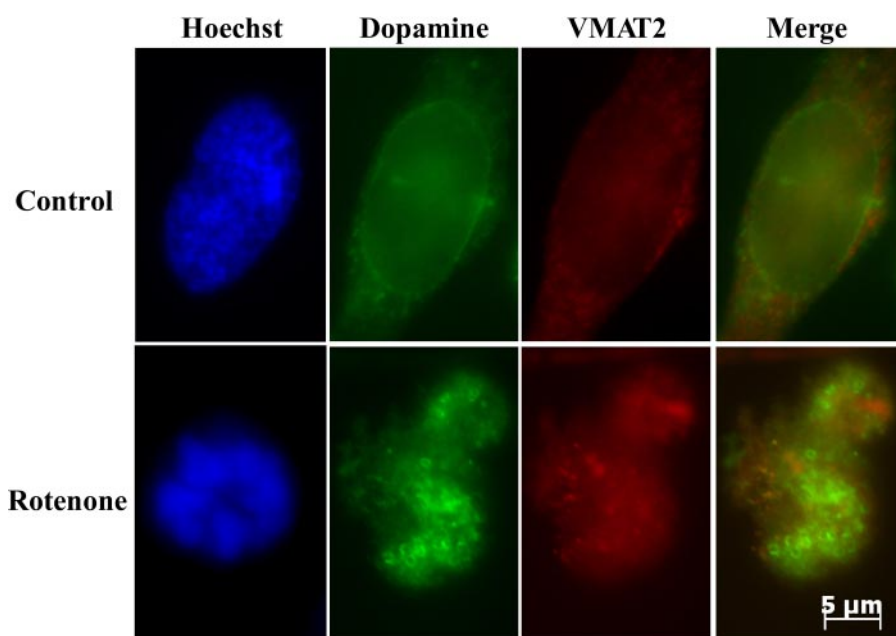

Fig. 1. Redistribution of intracellular dopamine in rotenone-treated SH-SY5Y cells. After SH-SY5Y cells had been treated with 0.4 μM rotenone for 8 h, the intracellular localizations of dopamine (green), VMAT2 (red), and the nucleus (blue; Hoechst staining) were detected immunocytochemically.

We have demonstrated that rotenone causes dopamine redistribution from vesicles to the cytosol (Watabe and Nakaki, 2007). Therefore, we next examined the effect of L-NAME on rotenone-induced dopamine redistribution from vesicles to the cytosol using an immunocytochemical technique. L-NAME inhibited the rotenone-induced dopamine redistribution from vesicles to the cytosol, as evidenced by the localization of dopamine being the same as that of VMAT2 (Fig. 3A). We confirmed this phenomenon using HPLC. As

shown in Fig. 3B, the dopamine amount was decreased in the vesicle fraction and increased in the cytosol fraction by rotenone treatment when cytosolic dopamine was prevented from leaking out of cells via DAT by GBR12935 (Fig. 3B). L-NAME yielded similar results for the distribution of dopamine (Fig. 3B). We reported previously that NAC had the same effects as L-NAME on rotenone-induced dopamine redistribution in SH-SY5Y cells (Watabe and Nakaki, 2007). These results suggest the nitration of the tyrosine residues of intracellular proteins to play a role in rotenone-induced dopamine redistribution and apoptosis.

TH nitration of tyrosine residues is known to be mediated by peroxynitrite or MPTP (Ara et al., 1998; Blanchard-Fillion et al., 2001). We examined whether the tyrosine residue of TH is also nitrated by rotenone treatment. Immunocytochemical examination, using anti-nitrotyrosine and TH antibodies, showed the localization of nitrotyrosine to be the same as that of VMAT2 in rotenone-treated cells and that both L-NAME and NAC inhibited the effects of rotenone (Fig. 4A). Immunoprecipitation, an alternate experimental technique, yielded similar results for the staining of TH and nitrotyrosine (Fig. 4, B and C).

Neuronal VMAT2 is essential to the redistribution of intracellular dopamine from the cytosol to vesicles. Because rotenone induced dopamine redistribution from vesicles to the cytosol via nitration, rotenone may directly affect VMAT2 via nitration of the tyrosine residues of VMAT2. To confirm this working hypothesis, we examined the nitration of tyrosine residues of VMAT2 in rotenone-treated cells. Immunocytochemical examination using anti-nitrotyrosine and VMAT2 antibodies showed the localization of nitrotyrosine to be the same as that of VMAT2 in rotenone-treated cells and that both L-NAME and NAC inhibited the actions of rotenone (Fig. 5A). The immunoprecipitation experiment yielded similar results for the staining of VMAT2 and nitrotyrosine (Fig. 5, B and C). Moreover, we examined whether inhibiting the vesicular dopamine uptake activity of VMAT2 with rotenone would lead to nitration of the tyrosine residues of VMAT2. To measure VMAT2 activity, we permeabilized rotenone-treated SH-SY5Y cells with SLO. SLO creates stable pores in the plasma membrane, which allow direct access of metabolites (Höltje et al., 2000; Brunk et al., 2006). Because dopamine uptake in SLO-permeabilized cells is completely inhibited by reserpine (Brunk et al., 2006), this uptake reflects the activity of VMAT2 but not that of DAT. Under these conditions, we examined VMAT2 activity by monitoring dopamine uptake into VMAT2-containing intracellular vesicles. Rotenone inhibited dopamine uptake by VMAT2, and this inhibition was reversed by pretreatment with either L-NAME or NAC (Fig. 6). These results indicate that rotenone-generated peroxynitrite shifts dopamine from vesicles to the cytosol via VMAT2 inhibition and thereby induces apoptosis.

VMAT2 immunoreactivity was reported recently to be obtained in Lewy bodies of the substantia nigra in patients with PD (Yamamoto et al., 2006). Therefore, we examined whether rotenone induces VMAT2 aggregation. Because thioflavin S fluorescence reveals the presence of protein fibrils, we performed double staining with VMAT2. Rotenone treatment showed an accumulation of aggregate-like formations in the stained image of VMAT2 colocalizing with thioflavin S (Fig. 7A). The increase in these morphologically altered cells is quantitatively significant (Fig.
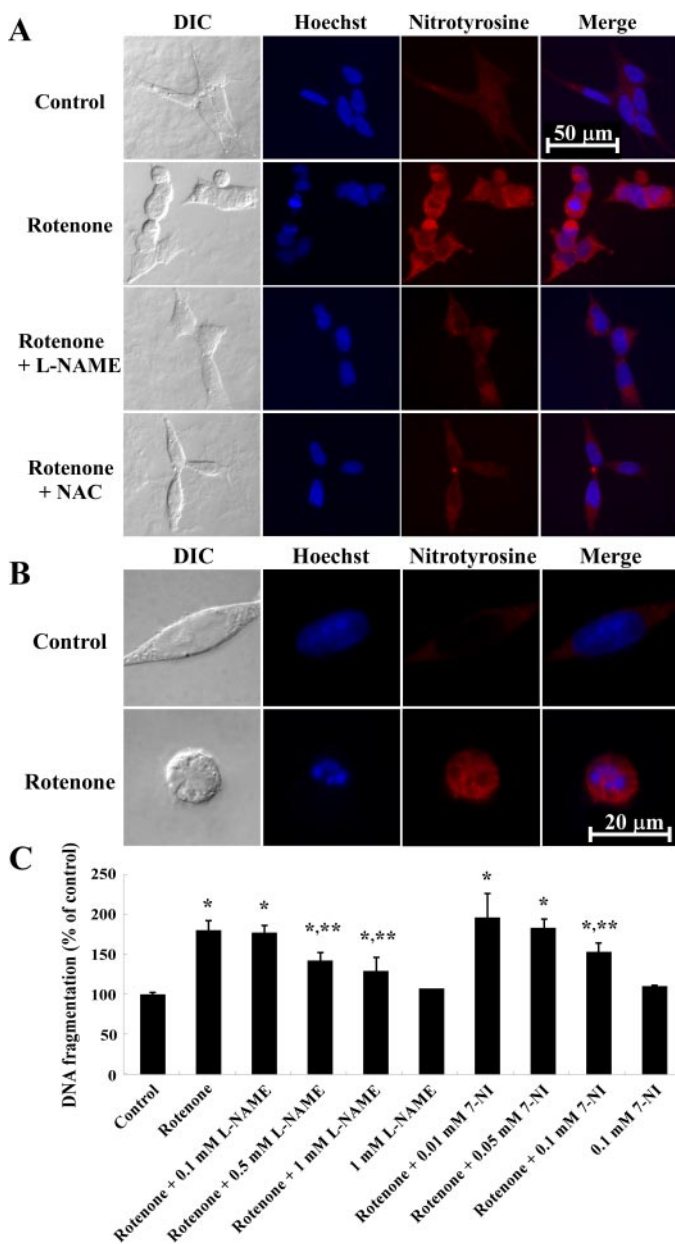

Fig. 2. Effects of NOS inhibitors on rotenone-induced apoptosis in SH-SY5Y cells. A, after SH-SY5Y cells had been treated with 0.4 μM rotenone for 8 h in the presence or absence of 1 mM L-NAME or 3 mM NAC, nitrotyrosine (red) and the nucleus (blue; Hoechst staining) were detected immunocytochemically. DIC, Nomarski differential interference contrast. B, SH-SY5Y cells were treated with 0.4 μM rotenone for 24 h, and nitrotyrosine (red) and the nucleus (blue; Hoechst staining) were detected immunocytochemically. C, SH-SY5Y cells were treated with 0.1 μM rotenone for 24 h in the presence or absence of L-NAME or 7-NI at various concentrations, and a DNA fragmentation assay was then performed. Results are presented as the mean of three independent experiments. *, $p < 0.05$ compared with control. **, $p < 0.05$ compared with rotenone.

7B). Moreover, we examined the effect of nitration on the rotenone-induced aggregate-like staining image of VMAT2. Treatment with both L-NAME and NAC inhibited the accumulation of these rotenone-induced VMAT2 aggregates in the fluorescent image (Fig. 7A). Immunoblotting, an alternate experimental technique, yielded similar results for the staining of VMAT2 and thioflavin S (Fig. 7C). These results indicate that the nitration signal inhibited VMAT2 protein activity and caused the accumulation of aggregate-like formations.

Discussion

We demonstrated previously that rotenone, which inhibits mitochondrial complex I and causes Parkinsonian motor dysfunction in rats, causes dopamine redistribution from vesicles to the cytosol during the apoptosis-induction process (Watabe and Nakaki, 2007). In the present study, we demonstrated that rotenone leads to the nitration and inactivation of VMAT2 and consequently causes dopamine redistribution from vesicles to the cytosol.

Peroxynitrite is a powerful oxidant and cytotoxic agent formed by the near-diffusion limited reaction between NO and superoxide (Koppenol et al., 1992). Peroxynitrite can damage DNA, membrane lipids, and mitochondria and has been shown to modify proteins at intrinsic methionine, tryptophan, and cysteine residues (Ischiropoulos and al-Mehdi, 1995; Beckman and Koppenol, 1996). Perhaps the best known property of peroxynitrite is its ability to nitrate free tyrosine and tyrosine residues in proteins (Ischiropoulos et al., 1992; Souza et al., 1999). Nitrotyrosine is a permanent marker of peroxynitrite attack on proteins (Beckman, 1994) and is found in postmortem brain samples of patients with PD (Good et al., 1998). Therefore, we examined changes in the amount of nitrotyrosine in rotenone-treated SH-SY5Y cells. Rotenone increased the nitrotyrosine amount, and this increase was suppressed by the NOS inhibitor L-NAME or the ROS scavenger NAC. Both L-NAME and the specific nNOS inhibitor 7-NI also suppressed rotenone-induced apoptosis. In an in vivo experiment, long-term rotenone administration to rats increased NO generation and induced
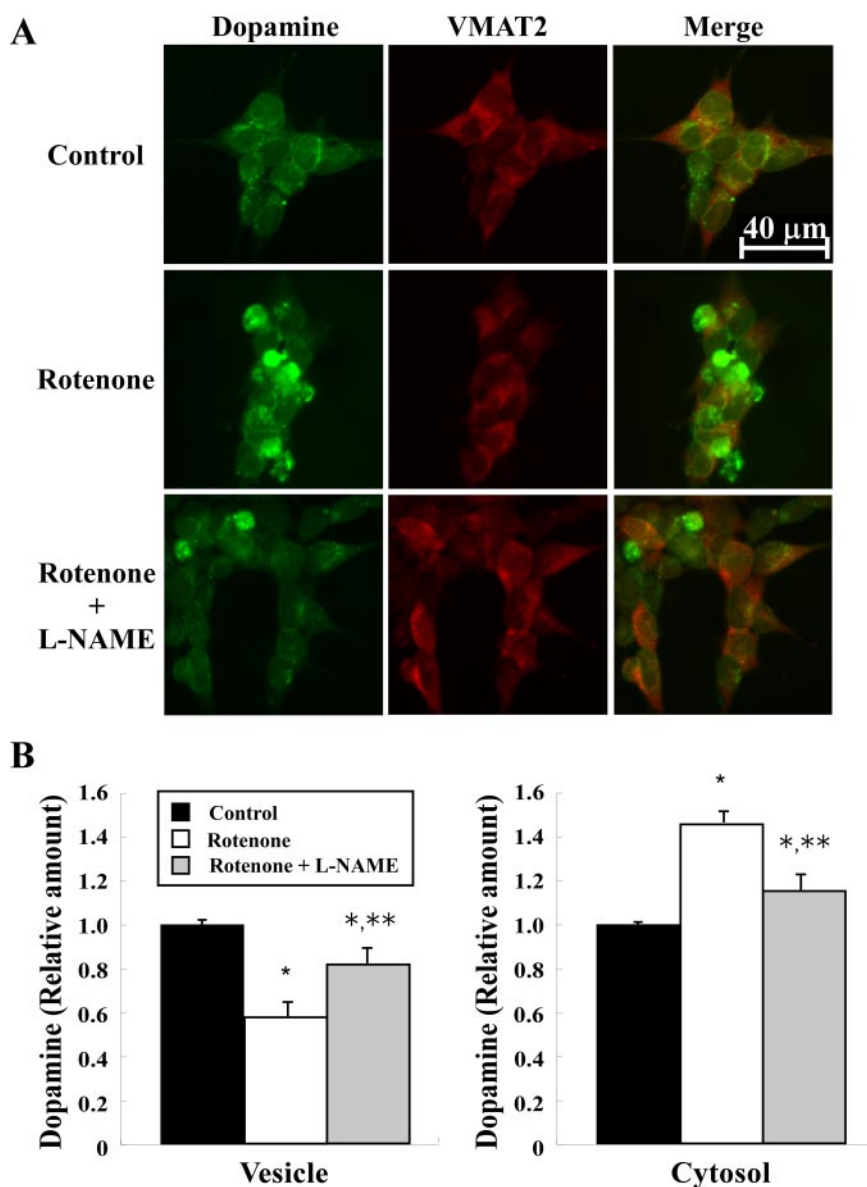

Fig. 3. Effect of NOS inhibitor on rotenone-induced redistribution of intracellular dopamine in rotenone-treated SH-SY5Y cells. A, after SH-SY5Y cells had been treated with 0.4 μM rotenone in the presence or absence of 1 mM L-NAME for 8 h, the intracellular localizations of dopamine (green) and VMAT2 (red) were examined by immunofluorescence microscopy. B, after SH-SY5Y cells had been treated with 0.4 μM rotenone and 1 mM L-NAME for 8 h in the presence of GBR12935, the vesicle (left) and cytosol (right) fractions were prepared, and the dopamine amounts in each fraction were measured. Results are presented as the mean of three independent experiments. *, $p < 0.05$ compared with control. **, $p < 0.05$ compared with rotenone.

nigra-striatal injury (He et al., 2003). Furthermore, we reported recently that NAC attenuated rotenone-induced DNA fragmentation in SH-SY5Y cells (Watabe and Nakaki, 2007). These results suggest nitration of the tyrosine residues of intracellular proteins to play a crucial role in rotenone-induced apoptosis.

In the experiments using peroxynitrite or MPTP, tyrosine nitration inactivated TH, which is a rate-limiting enzyme in dopamine synthesis, and is found in α-synuclein, a major component of Lewy bodies (Kuhn et al., 2004). Rotenone dose-dependently increased DNA fragmentation, which is a marker of apoptosis induction, in human dopaminergic SH-SY5Y cells, and during this process, the amount of TH was unchanged, as shown previously (Watabe and Nakaki, 2004). We have also shown that rotenone causes dopamine redistribution from vesicles to the cytosol and ultimately decreases intracellular dopamine levels in SH-SY5Y cells (Watabe and Nakaki, 2007). In the present study, rotenone caused nitration of the tyrosine residues of TH. Because nitration of one or more tyrosine residues of TH was temporally associated with the loss of enzymatic activity, one of the reasons for rotenone-decreased dopamine levels might be TH inactivation via nitration. However, reduced dopamine levels alone cannot trigger apoptosis in the manner of reserpine treatment (Watabe and Nakaki, 2007). Furthermore, we have shown inhibition of dopamine synthesis to attenuate rote-

none-induced DNA fragmentation in SH-SY5Y cells (Watabe and Nakaki, 2007). These results suggest that loss of the enzymatic activity of TH alone, via nitration, cannot account
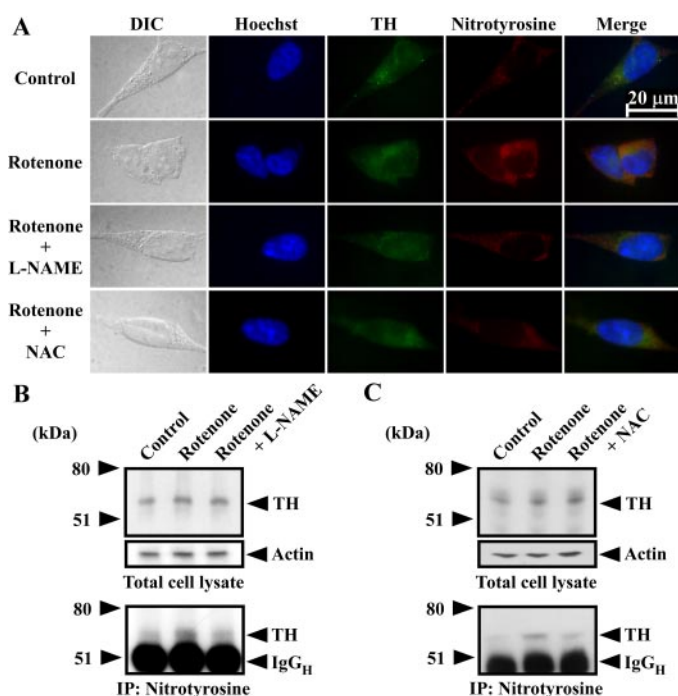

Fig. 5. Induction of nitration of the tyrosine residue of VMAT2 on rotenone-treated SH-SY5Y cells. A, SH-SY5Y cells were treated with 0.4 μM rotenone for 8 h in the presence or absence of 1 mM L-NAME or 3 mM NAC. VMAT (green), nitrotyrosine (red), and the nucleus (blue; Hoechst staining) were detected immunocytochemically. B, SH-SY5Y cells were treated with 0.4 μM rotenone for 8 h in the presence or absence of 1 mM L-NAME. Using total cell lysates (top) and immunoprecipitates obtained with anti-nitrotyrosine antibody (bottom), immunoblotting analysis was performed using anti-VMAT2 and antiactin antibodies. C, SH-SY5Y cells were treated with 0.4 μM rotenone for 8 h in the presence or absence of 3 mM NAC. Using total cell lysates (top) and immunoprecipitates obtained with anti-nitrotyrosine antibody (bottom), immunoblotting analysis was performed using anti-VMAT2 and anti-actin antibodies.
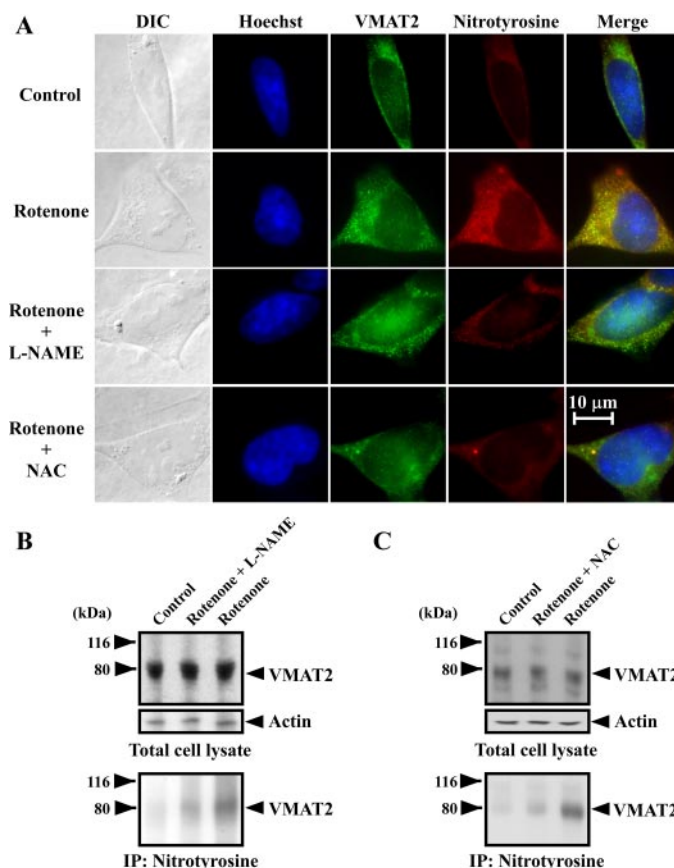

Fig. 4. Induction of nitration of tyrosine residues of TH on rotenone-treated SH-SY5Y cells. A, SH-SY5Y cells were treated with 0.4 μM rotenone for 8 h in the presence or absence of 1 mM L-NAME or 3 mM NAC. TH (green), nitrotyrosine (red), and the nucleus (blue; Hoechst staining) were detected immunocytochemically. B, SH-SY5Y cells were treated with 0.4 μM rotenone for 8 h in the presence or absence of 1 mM L-NAME. Using total cell lysates (top) and immunoprecipitates obtained with anti-nitrotyrosine antibody (bottom), immunoblotting analysis was performed with anti-TH and antiactin antibodies. C, SH-SY5Y cells were treated with 0.4 μM rotenone for 8 h in the presence or absence of 3 mM NAC. Using total cell lysates (top) and immunoprecipitates obtained with anti-nitrotyrosine antibody (bottom), immunoblotting analysis was performed using anti-TH and antiactin antibodies. Heavy chain indicates the IgG heavy chain.
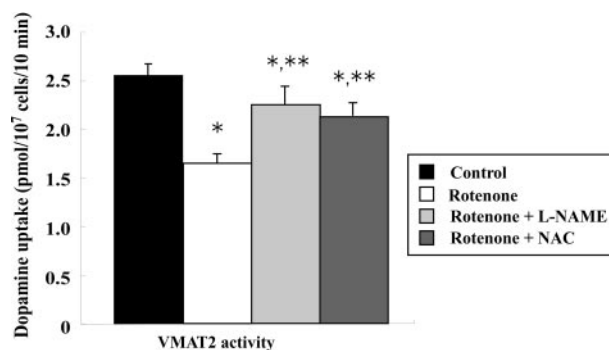

Fig. 6. Inhibition of VMAT2-mediated dopamine uptake by rotenone. After SH-SY5Y cells had been treated with 0.4 μM rotenone in the presence or absence of 1 mM L-NAME or 3 mM NAC for 8 h, the cells were permeabilized with SLO, and dopamine uptake was measured. Results are presented as the mean of three independent experiments. $*$, $p < 0.05$ compared with control. $**$, $p < 0.05$ compared with rotenone.

for rotenone-induced apoptosis. On the other hand, we also have shown that rotenone induced an increase in the amount of α-synuclein during the process of apoptosis (Watabe and Nakaki, 2004). The aggregation of α-synuclein has been suggested to be important in the etiology of PD. Because the nitration of α-synuclein in PD provides evidence directly linking oxidative and nitrative damage to the onset and progression of neurodegenerative synucleinopathies (Giasson et al., 2000), rotenone may cause the nitration of α-synuclein under our experimental conditions.

Dopamine is a neurotransmitter under physiological conditions but also acts as a neurotoxin participating in the neurodegenerative process. The mechanism underlying dopamine neurotoxicity is strongly linked to oxidative metabolism. Dopamine can be oxidized in vitro or in vivo to form ROS, free radicals, and quinones, which are probably covalently incorporated into a variety of molecules including lipids, proteins, and nucleic acids. Because vesicular dopamine is protected from oxidative assault, in the neurodegenerative process, dopamine needs to be expelled from vesicles, thereby becoming vulnerable to ROS and enzymatic metabolism. In fact, rotenone induces dopamine redistribution from vesicles to the cytosol in SH-SY5Y cells (Watabe and Nakaki, 2007). On the other hand, rotenone inhibits complex I and generates ROS in mitochondria (Li et al., 2003). We have also demonstrated rotenone to cause ROS generation in SH-SY5Y cells (Watabe and Nakaki, 2007). Therefore, it is possible that oxidative cytosolic dopamine acts as a neurotoxin and causes this neurodegeneration. Furthermore, it is interesting that NAC (Watabe and Nakaki, 2007) and L-NAME (Fig. 3) inhibited both dopamine redistribution and apoptosis induction.

In the central nervous system, VMAT2 is the only transporter that moves cytosolic dopamine into synaptic vesicles for storage and subsequent exocytotic release. Therefore, to elucidate how rotenone causes dopamine redistribution from vesicles to the cytosol, we focused on VMAT2 as a key factor in the neurodegenerative process. In particular, we examined the relation between nitration and activation of VMAT2 because dopamine redistribution was inhibited by both L-NAME and NAC. Using an immunocytochemical technique, the nitration of tyrosine residues via VMAT2 was caused by rotenone. However, the merged images with multiple antibodies must be carefully interpreted, even in light of the confocal imaging data. Therefore, using immunoprecipitation as another technique, we established that the nitration of tyrosine residues via VMAT2 was caused by rotenone and the reduced dopamine uptake activity of VMAT2. A previous study demonstrated that tyrosine 434 of VMAT2 plays an important role in dopamine affinity and the activity of dopamine transport (Finn and Edwards, 1997). The replacement of tyrosine 434 with alanine increases the affinity of VMAT2 for dopamine and reduces the rate of dopamine transport. Therefore, rotenone may cause nitration of tyrosine 434 of VMAT2 and thereby inhibit dopamine transport activity. We conclude that rotenone-generated ROS and NO reduce dopamine uptake by VMAT2 via nitration, induce dopamine redistribution from vesicles to the cytosol, modify the cytosolic dopamine molecule, and trigger apoptosis of dopaminergic SH-SY5Y cells.

Physiological concentrations of dopamine do not induce degenerative processes in the brain and have even been shown to activate the expression of cell survival genes and proteins (Weinreb et al., 2003). In contrast, numerous studies have shown that at higher concentrations, dopamine can cause cell death both in vivo and in cell cultures. Even at physiological intracellular concentrations of dopamine, redistribution from vesicles to the cytosol results in dopamine toxicity. Therefore, the dopamine redistribution produced by VMAT2 inhibition via nitration might be a common step underlying the mechanisms of dopaminergic neuronal toxins known to induce oxidative stress.

Lewy bodies are cytoplasmic inclusions present in nerve cell bodies and processes, and are the pathological hallmark
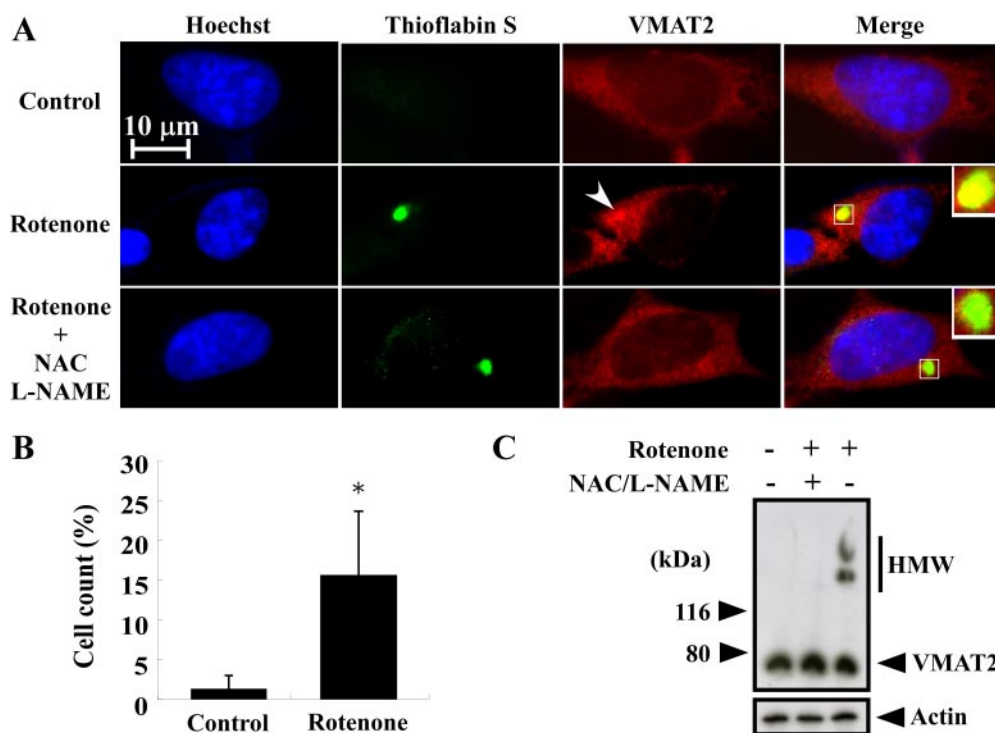
Fig. 7. Effect of nitration on rotenone-induced intracytosolic accumulation of aggregate-like formations in the stained image of VMAT2. SH-SY5Y cells had been treated with 0.04 μM rotenone in the presence or absence of 1 mM L-NAME and 3 mM NAC for 72 h. A, thioflavin S (ThioS; green), VMAT2 (red), and the nucleus (blue; Hoechst staining) were detected immunocytochemically. Arrowhead indicates aggregated VMAT2. A magnified area is shown in the inserted square. B, cells positive for both thioflavin S and VMAT2 were counted. ∗, $p < 0.05$ compared with control. C, immunoblotting analysis was performed using anti-VMAT2 and anti-actin antibodies. HMW, high molecular weight.

of PD and diffuse Lewy body disease. Viewed from an ultrastructural perspective, Lewy bodies are composed mainly of filamentous, vesicular, and granular materials. VMAT2 immunoreactivity has also been demonstrated in Lewy bodies and Lewy neuritis in the substantia nigra of patients with PD (Yamamoto et al., 2006). Therefore, using an in vitro model of human dopaminergic SH-SY5Y cells, we found VMAT2 to be inactivated by the nitration signal and established that the accumulation of aggregate-like formations of VMAT2 was caused by nitration. Both α-synuclein and TH, which are the main constituents of Lewy bodies, are inactivated by nitration and consequently form aggregates resembling intracytosolic inclusions (Nakashima and Ikuta, 1984; Spillantini et al., 1997; Giasson et al., 2000; Blanchard-Fillion et al., 2001). Therefore, the nitration signal might be a common step underlying the formation of protein-aggregated intracytosolic inclusions such as Lewy bodies in PD and diffuse Lewy body disease.

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