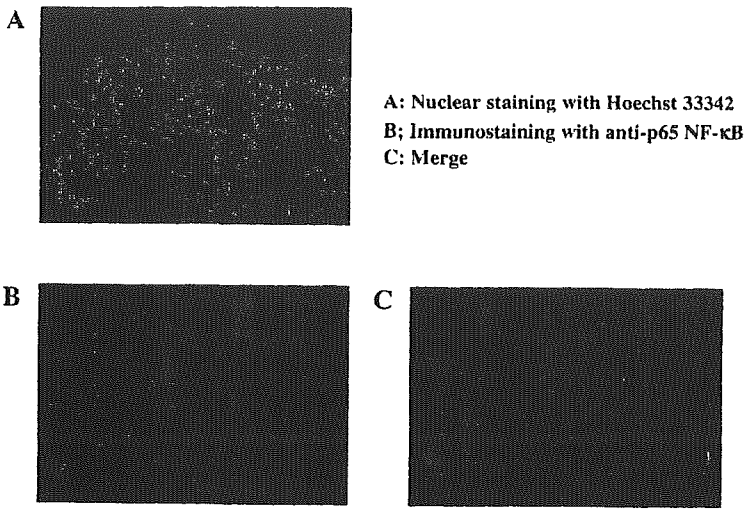

Figure 7 Nuclear translocation of p65 NF-κB subunit by the treatment with rasagiline. SH-SY5Y cells were treated with 0.1 μM of rasagiline for 3 hr and fixed in paraformaldehyde. The sample was stained with Hoechst 33342 nuclear staining (A) and immunostaining using anti-p65 antibody (B). (C) shows the merge of (A) and (B).

The activation of NF-κB and the increased binding activity were examined also by ELISA, using NF-κB p65 transcription assay kit according to Kretz-Remy et al. (42). The principle of this assay is to measure the binding of activated NF-κB p65 to an oligonucleotide containing the NF-κB consensus-binding site. Rasagiline increased the binding activity of NF-κB p65 to the oligonucleotides and it was competitively inhibited by pretreatment with wild oligonucleotide containing the NF-κB binding site, but not the mutated one, indicating the selective binding to the NF-κB binding site.

The involvement of phosphorylation of IκB, an inhibitory subunit on the activation of NF-κB, was studied by use of sulfasalazine, an inhibitor of IκB kinase as summarized in Table 1. Also NF-κB binding assay showed that sulfasalazine suppressed the rasagiline-induced increase in the binding capacity, again suggesting the involvement of IκB kinase–NF-κB axis. In addition, Western blot analysis of the

subcellular fractions of rasagiline-treated cells demonstrated that sulfasalazine reduced nuclear translocation of activated p65 subunit.

Sulfasalazine abolished the increase of mRNA of *bcl-2* and *bcl-xL* as in the case with GDNF, suggesting the involvement of NF-κB transcription factor in the induction of neuroprotective genes in common (Fig. 5B).

II.D. Gene Expression by Rasagiline Detected by DNA Array Analyses

SH-SY5Y cells were treated with 0.1 μM of rasagiline for 6, 12, and 24 hr and mRNA was extracted and reverse-transcripted with biotylated dUTP (Roche Diagnostics) and gene-specific primer mixture reported as the manufacture's instruction (Takara Bio Co., Otsu, Japan). The probes were hybridized to a cDNA expression array membrane containing more than 2000 genes related to apoptosis, cell survival, and transcription. The relative expression level of a given mRNA was assessed by normalizing to a housekeeping gene, β-actin, provided on the membrane and comparing to the control values obtained by the cells without treatment of rasagiline.

The gene induction was widely surveyed by gene array analysis system to compare the level of mRNA relating apoptosis-survival signal in the cells with or without the treatment of rasagiline (Table 2). Rasagiline increased mRNA of the genes relating mitochondria and ATP synthesis, ubiquitin–proteasome system and Bcl-2 in first 6 hr and then, genes relating signal transduction and transcription, including a series of kinases and NF-κB, were increased after 24 hr.

III. DISCUSSION

This paper reports that rasagiline induces neuroprotective genes in SH-SY5Y cells through the activation of transcription factor NF-κB. Rasagiline is a selective inhibitor of MAO-B, but its neuroprotective effect cannot be ascribed to MAO inhibition, because SH-SY5Y cells do not contain MAO-B. Recent

Table 2 Gene Induction in SH–SY5Y Cells by Rasagiline

Cells incubated with rasagiline for 6 hr	12 hr	24 hr
Metabolism and ATP synthesis		
Cytochrome c oxidase	ATP synthase	*Cell signaling*
NADH-coenzyme Q reductase	Cytochrome c oxidase	Tumor protein 53-binding protein
ATP synthase	ATP binding protein	PTK2 protein tyrosine kinase 2
Aconitase		MAP kinase 6
		LPS-induced TNF-alpha factor
		TNF receptor member 6
		PTK2 protein tyrosin kinase 2
		MAP kinase kinase 6
		TNFRSF-interacting serine/threonine kinase
		Tumor protein 53-binding protein
		Cyclin-dependent tyrosine kinase 5 (p35)
		Neurotrophic tyrosine kinase receptor
Apoptosis		
Bcl-2	Apoptosis inhibitor 2	Bcl-2
	Apoptosis inhibitor	
	Bcl-2 like	
	Neuronal apoptosis inhibitory protein	
Transcription		
	Mitochondrial transcription factor A	Transcription factor Dp-1, Dp-2
	Transcription elongation factor B	TRAF-associated NFkB activator
		E2F transcription factor 5, 3
		P130-binding NFkB
Intracellular protein degrading		
Proteasome subunit, b type 3, 1, 7, 5	Ubiquitin-conjugating enzyme E2N	
Ubiquitin fusion degradation 1 like	Ubiquitin fusion degradation 1 like	
	Proteasome subunit, b type 3, 1	

study revealed that rasagiline and other structurally related propargylamines rescue neurons from apoptosis by inhibiting the induction of mPT and the reduction of $\Delta\Psi_m$ the critical step to initiate apoptosis signal. Rasagiline was found to inhibit PT induced by an endogenous neurotoxoin *N*M(*R*)Sal in isolated mitochondria suggesting its direct interaction to the mitochondrial protein (23). Tatton et al. (43) reported that (−) deprenyl rescued neuronal differentiated PC12 cells from apoptosis induced by serum deprivation. They augmented that nuclear translocation of GAPDH inhibited the transcription of *bcl-2* and *bcl-xL* and resulted in mPT, and that (−) deprenyl interfered GAPDH polymerization into the tetramers, which was essential for the nuclear translocation. However, we found that nuclear translocation of GAPDH was a downstream signal of the induction of mPT (44). In addition, we showed that rasagiline did not suppress the decrease, but even increased the transcription of *bcl-2* and *bcl-xL*. NF-κB is one of the most important transcriptional factor, which regulates the cell death-survival signal and is suggested to be involved in the activation of prosurvival genes in neuronal cells in the preconditioning model of ischemia and amyloid β protein (45,46). Rasagiline activates NF-κB, which was antagonized by sulfasalazine, an inhibitor of IκB kinase. Considering that sulfasalazine abolishes the increase of GDNF, *bcl-2*, and *bcl-xL*, these proteins are induced by IκB kinase–NF-κB pathway. Gene array study of rasagiline-treated cells reveals that rasagiline increases the genes relating mitochondrial energy synthesis, apoptosis, transcription, and proteasome system by a time course way. At present, the mechanism how rasagiline activates NF-κB transcription factor is not fully clarified, but our recent results suggest that there may be a signal transduction from mitochondria to a kinase, which activates NF-κB pathway. The study to find out the target molecule of rasagiline may give us a clue to develop new neuroprotective drugs that intervene the transcription of the cell death-regulating genes in the central nervous system.

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16

Oxidative Stress in Mitochondria: The Involvement in Neurodegenerative Diseases

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ABSTRACT

In mitochondria, oxidative phosphorylation and enzymatic oxidation of biogenic amines by monoamine oxidase produces reactive oxygen and nitrogen species, which may account for neuronal cell death in neurodegenerative disorders, including Parkinson's and Alzheimer's disease. In these disorders, inclusion body composed of oxidation-modified proteins and lipids is detected specifically for distinct diseases, such as the Lewy body for Parkinson's disease. The relationship between mitochondrial dysfunction, increased oxidative stress, accumulation of oxidation-modified protein, and final cell death of definite neurons in the brain remains to be clarified. In this paper, we review our recent results on interaction among these factors in neurons, using a cellular model of apoptosis induced by peroxynitrite-generating *N*-Morpholino sydnonimine (SIN-1) and an inhibitor of complex I, rotenone in human dopaminergic SH-SY5Y cells. In control cells, 3-nitrotyrosine-containing protein produced by peroxynitrite was detected, suggesting that neurons exist in a state of constant oxidative stress. *N*-Morpholino sydnonimine induced apoptosis and reduction in ATP level, which is, increased further by an inhibitor of proteasome, carbobenzoxy-L-isoleucyl-γ-*t*-butyl-glutamyl-L-alanyl-L-leucinal (PSI). The subunits of mitochondrial complex I were found to contain 3-nitrotyrosine, suggesting that peroxynitrite prefers these enzymes. In addition, rotenone induced mitochondrial dysfunction, and accumulation and aggregation of protein modified with acrolein, an aldehyde product of lipid peroxidation. Rotenone treatment reduced the enzymatic activity of the proteasome system, a major organelle in the degradation of oxidation-modified protein, and it was due to the oxidative modification of 20S β subunit of the proteasome. These results are discussed in relation to the interaction between mitochondrial dysfunction, oxidative stress, and proteasome inactivation, resulting in neuronal cell death in neurodegenerative disorders, such as Parkinson's and Alzheimer's disease.

I. OXIDATIVE STRESS AND MODIFIED PROTEIN AS THE MARKER

Oxidative stress has been proposed to induce neuronal death in aging and age-associated disorders (1,2), and mitochondria are a major source of reactive oxygen and nitrogen species (ROS–RNS). The superoxide anion radical generated by oxidative phosphorylation in the mitochondria is one of the most potent ROS and reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), whereas oxidation of biogenic amines by monoamine oxidase in mitochondrial outer membrane produces hydrogen peroxide. Mitochondria are now considered to play a pivotal role in apoptosis (3), which emerges as a common death type of neurons in neurodegenerative disorders, including Parkinson's (PD) and Alzheimer's diseases (AD) (4,5). The role of mitochondria in the process of apoptotic commitment is recognized. In mitochondria, impairment of energy charge and redox, permeability transition (PT), disruption of membrane potential, $\Delta\Psi m$, and release of cytochrome *c* are observed prior to the fragmentation of nuclear DNA, a hallmark of apoptotic morphological features.

Neurodegenerative disorders are characterized by a decline of specified neurons associated with protein deposits typical for each disease. In PD, dopamine neurons in the substantia nigra degenerate progressively with the formation of the Lewy bodies (LB). The pathogenesis of PD remains unknown, and the gene responsible for the sporadic cases has not been identified. PD is considered to represent the final outcome of various genetic and environmental interactions. The vulnerability of dopamine neurons is a consequence of the increased generation of ROS and RNS, reduced antioxidant capacity, high content of iron and dopamine, and possible defect in mitochondrial function. ROS and RNS generated in mitochondria modify bioactive molecules, such as lipids, proteins, DNA, and carbohydrates, either directly or indirectly with peroxidation products of lipids or carbohydrates. Several kinds of modified bioactive molecules have been proposed as markers of oxidative modification by ROS and RNS, as summarized in Table 1. Hydroxyl radicals

Table 1 Oxidative Modification of Protein

Direct modification	Secondary modification
Polymerization (cross reaction)	Modification by lipid peroxidation
Aggregation	Aldehydes
Fragmentation	4-Hydroxynonenal
	Acrolein
Inactivation or activation of enzymes	Malondialdehyde
	Hydroperoxide
Modification of amino acids	Carbonyl production
3-Nitrotyrosine	
Dityrosine	Modification by glycosylation
	Aldehydes
Carbonyl production	Carbonyls

modify tyrosine, phenylalanine, tryptophan, histidine, methionine, and cysteine residues as preferred targets. Under anaerobic conditions, the hydroxyl radicals promote protein–protein crosslinking through –S–S– and –tyrosyl-tyrosyl– (dityrosine) bonding, and under aerobic conditions, peroxyradicals induce fragmentation of the polypeptide chain. In addition, proline, arginine, and lysine are particularly sensitive to metal-catalyzed oxidation and are converted to carbonyl derivatives.

Oxidative modification produces aggregated and crosslinked proteins, which are resistant to proteolytic degeneration and are difficult to be removed from the cells. Accumulation of the modified proteins may impact on a variety of cellular pathways by changing the enzymatic, regulatory, and transporting potencies of cellular specific protein, in addition to taking up space in limited cellular volume. The level of the oxidized protein may reflect the balance between the generation of ROS–RNS and degradation of modified protein, in which the ubiquitin–proteasome system plays a key role (6).

One of the most active RNS is peroxynitrite ($ONOO^-$) (7), which is unstable, but its protonated peroxynitrous acid (ONOOH) is extremely reactive (8), which generates hydroxyl radical by homolytic cleavage (9). The main targets of nitration are sulfhydryl and hydroxyl residues in cysteine,

methionine, phenylalanine, and tyrosine. It inactivates the membrane function and key enzymes (see reviews 7, 10). As shown in Fig. 1, 3-nitrotyrosine (3-NT) is synthesized by the nitration of tyrosine residues in protein and a marker for the oxidative stress induced by peroxynitrite in vivo (11). 3-Nitrotyrosine containing protein (3-NT protein) was detected in atherosclerosis (12) and neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS) (7), AD, (13,14), and PD (15). Figure 2 shows the immuno-histochemical detection of 3-NT protein in pyramidal hippocampal neurons, using anti-3-NT protein antibody (16). Another oxidation product of tyrosine is dityrosine, which is produced from free and protein-bound tyrosine in the presence of hydrogen peroxide

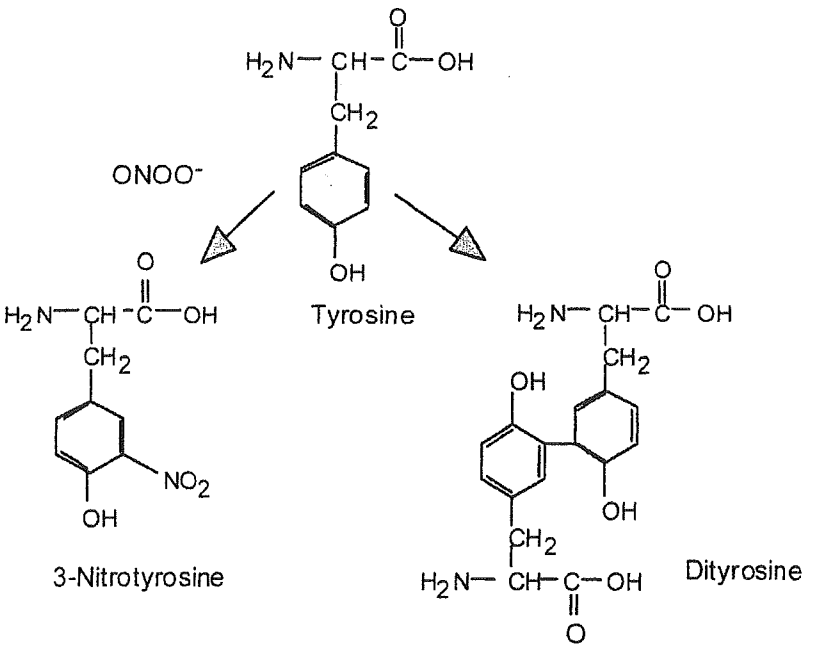

Figure 1 Modification of tyrosine residue in protein by NOS and RNS. Peroxynitrite modifies tyrosine residues to 3-nitrotyrosine in protein, and hydrogen peroxide and peroxidase, and irradiation produces tyrosyl radicals, yielding dityrosine with tyrosine residues in protein or free tyrosine.

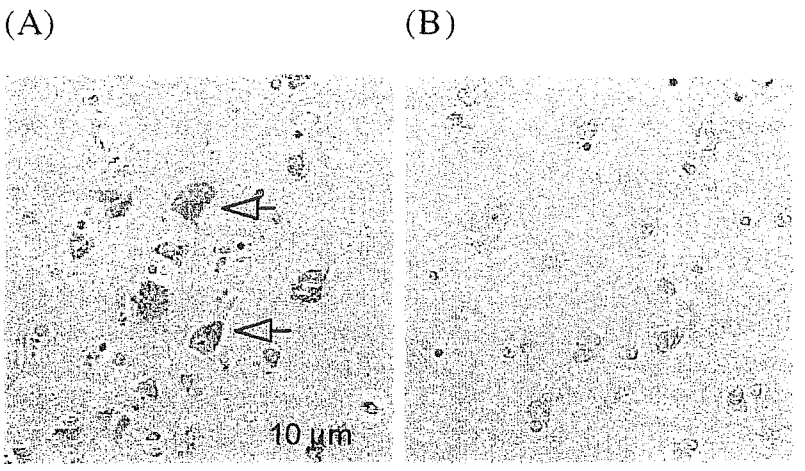

Figure 2 Immuno-histochemical detection of 3-NT-containing protein in lipofuscin in pyramidal hippocampal neurons. The brain was obtained from a 72-year-old male patient without neurological or psychiatric disorders. The tissue samples were incubated with (A) anti-3-NT protein antibody (diluted 1:200 with bovine serum albumin) or (B) bovine serum albumin alone, followed by the treatment of peroxidase-labeled antirabbit IgG. Lipofuscin stained was observed as brown granule as indicated by arrows.

and myeloperoxidase (17) (Fig. 1), and is detected in atherosclerotic plaques (18) and lipofuscin pigments in the aged human brain (19).

On the other hand, lipid peroxidation generates various reactive aldehydes, including 4-hydroxynonenal (4-HNE) and acrolein as shown in Fig. 3 (20). 4-Hydroxynonenal reacts with sulfhydryl and amino groups and leads to inactivation of DNA polymerases, dehydrogenases, and various transporters, and also to cell cycle arrest and apoptosis. Proteins modified with 4-HNE and malondialdehyde were detected in nigro-striatal dopamine neurons in PD (21), neurofibral tangles in AD (22,23), and the spinal cord of ALS patients (24). Acrolein, CH_2=CH–CHO, is ubiquitously generated in the biological system and is the most reactive α,β-unsaturated aldehyde product of lipid peroxidation. It is incorporated into proteins easily and accumulates as protein adducts

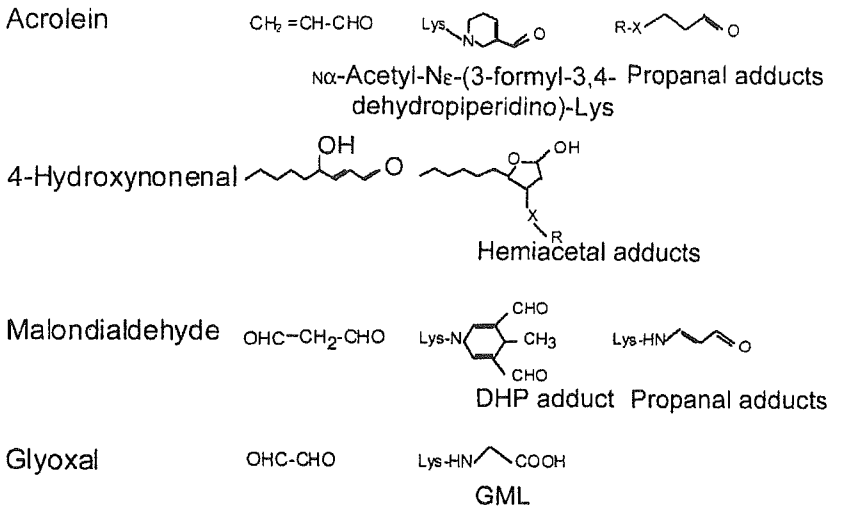

Figure 3 Modification of protein by aldehyde products of lipid peroxidation. Aldehyde products of lipid and carbohydrate peroxidation, acrolein, 4-hydroxynonenal, malondialdehyde, and glycol, modify lysine residues in proteins. DHP, dihydropyridine, GML, glyoxal modified lysine.

after reacting with lysine and histidine residues by forming Michaelis-type acrolein–amino acid complexes (25). Acrolein-modified protein was detected in oxidized low-density lipoproteins (26) and the brain of patients with AD (27). Figure 4 shows the histochemical staining of the substantia nigra in a parkinsonian brain with an antibody against 4-HNE-modified protein. Dopamine neurons containing neuromelanin from parkinsonian patients were stained more markedly than those in normal control and nondopaminergic cells. These results indicate that the oxidative stress increases markedly in nigro-striatal dopamine neurons of a parkinsonian brain.

II. MITOCHONDRIAL COMPLEX I SUBUNITS ARE NITRATED BY $ONOO^-$

In the brain, NO has been considered to be produced in microglia and astrocytes and transported to neurons, where it

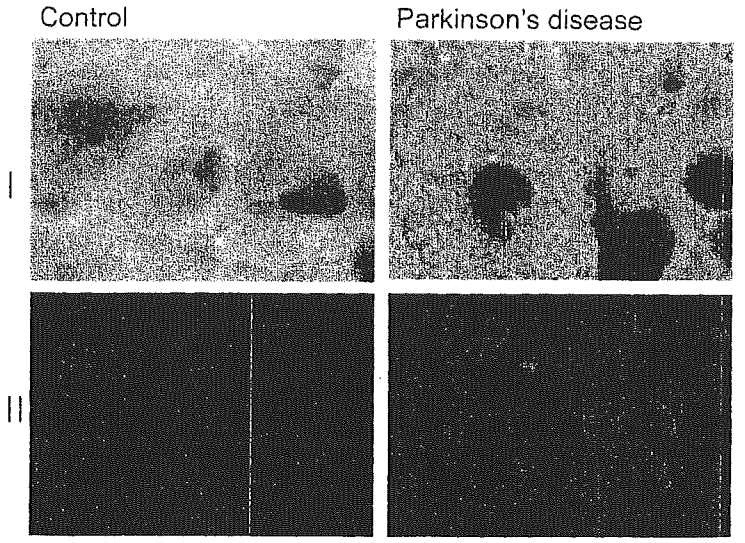

Figure 4 Occurrence of 4-HNE-modified protein in dopamine neurons in substantia nigra of the brain from the patient with Parkinson's disease. Only in the substantia nigra of the brain from the parkinsonian patient, but not from control, dopamine neurons containing neuromelanin are positively stained with antibody against 4-HNE modified protein. Cells other than dopamine neurons are not stained with the antibody.

reacts with the superoxide yielding $ONOO^-$. However, SH-SY5Y cells produce NO and $ONOO^-$ in situ, as confirmed by the use of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (28) and inhibitors of nitric oxide synthase (NOS); H_2DCFDA is cleaved into 2′,7′-dichlorofluorescein by hydroxyl radical and $ONOO^-$; and NOS inhibitors, N^5-(l-iminoethyl) -L-ornithine (L-NIO) and N^5-nitro-L-arginine methyl ester (L-NAME), reduced DCF to about a half.

Using an antibody against the 3-NT protein (16), nitrated proteins were detected in human dopaminergic SH-SY5Y cells. The lysate from these cells was subjected to western blot analysis as shown in Fig. 5A, and the molecular weight of major 3-NT proteins was estimated to be 33, 21, 15, and 11 kDa. The nitrated protein bands were detected even in control under physiological conditions, suggesting that the cells were under

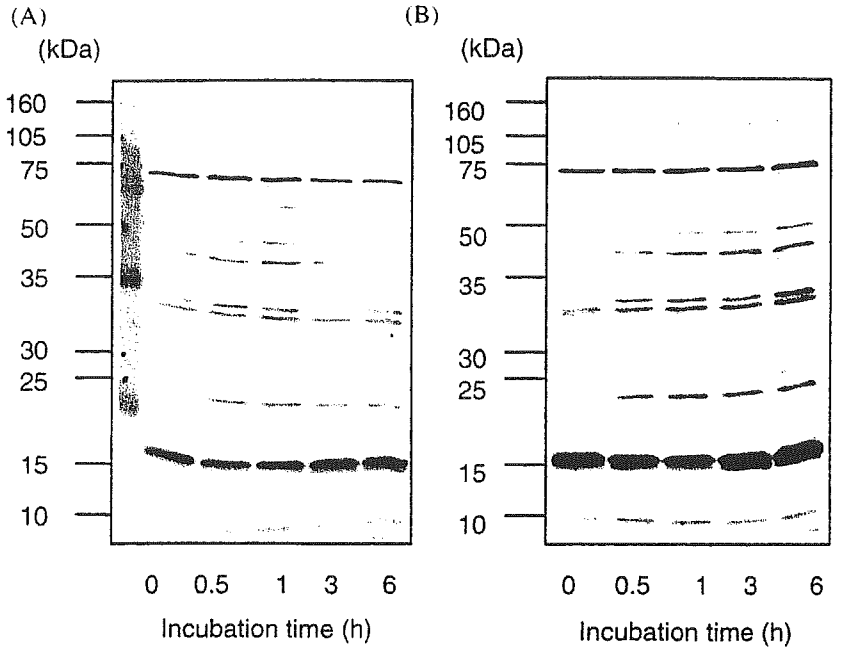

Figure 5 Formation of 3-NT protein in SH-SY5Y cells and the effects of peroxynitrite-generating SIN-1 and a proteasome inhibitor, PSI. (A) Cells were treated with 250 μM of SIN-1, then applied to the immunoblotting with antibody against 3-NT proteins. After the treatment with SIN-1, the intensity of 3-NT proteins increased, but the number did not change markedly. At the left lane in A, the molecular markers are shown. (B) The cells were treated with SIN-1 (250 μM) in the presence a proteasome inhibitor, PSI (10 μM). 3-Nitrotyrosine protein increased in amount according to the incubation time.

constant oxidative stress. However, the cells are intact in growth and proliferation, suggesting the functioning of an active mechanism to eliminate modified protein from the cells. The mitochondrial fraction of the SH-SY5Y cells was the one most intensively stained with the anti-3-NT antibody, and was subjected to western blot analysis using antibodies against 3-NT protein, and against mitochondria complex I, II, III, and IV (29). Some of the nitrated proteins were identified to be