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

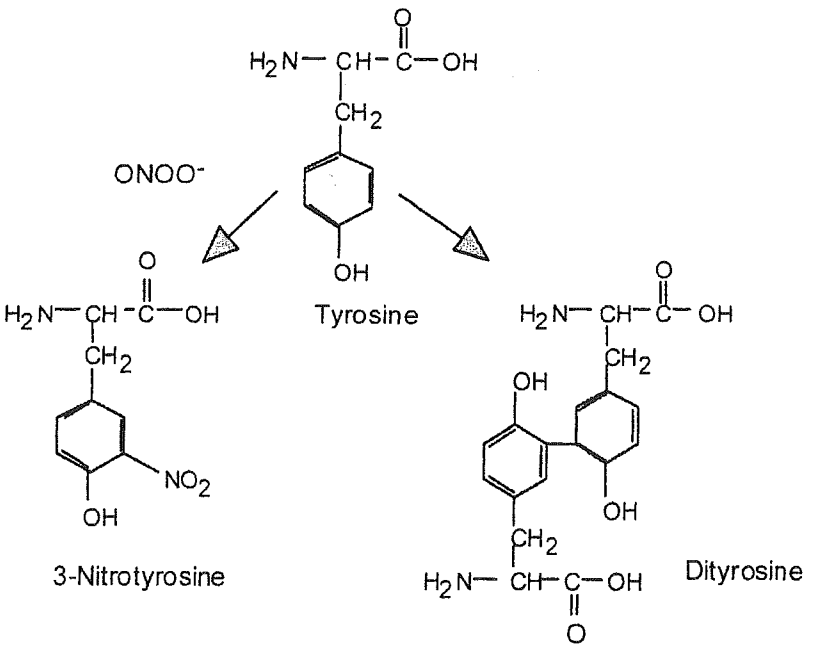

Figure 1 Modification of tyrosine residue in protein by NOS and RNS. Peroxynitrite modifies tyrosine residues to 3-nitrotyrosine in protein, and hydrogen peroxide and peroxidase, and irradiation produces tyrosyl radicals, yielding dityrosine with tyrosine residues in protein or free tyrosine.

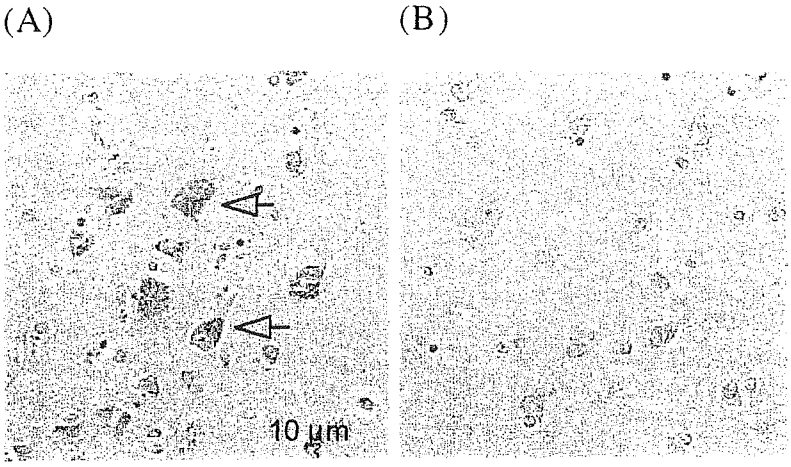

Figure 2 Immuno-histochemical detection of 3-NT-containing protein in lipofuscin in pyramidal hippocampal neurons. The brain was obtained from a 72-year-old male patient without neurological or psychiatric disorders. The tissue samples were incubated with (A) anti-3-NT protein antibody (diluted 1:200 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

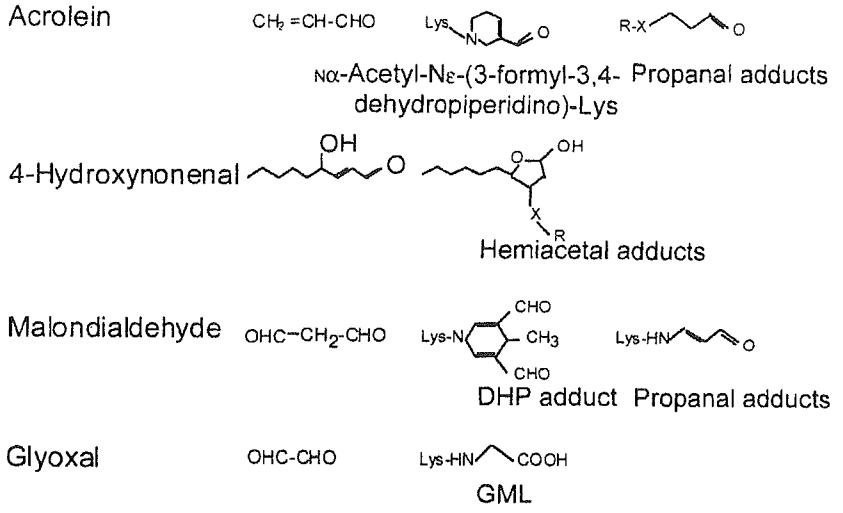

Figure 3 Modification of protein by aldehyde products of lipid peroxidation. Aldehyde products of lipid and carbohydrate peroxidation, acrolein, 4-hydroxynonenal, malondialdehyde, and glycol, modify lysine residues in proteins. DHP, dihydropyridine, GML, glyoxal modified lysine.

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

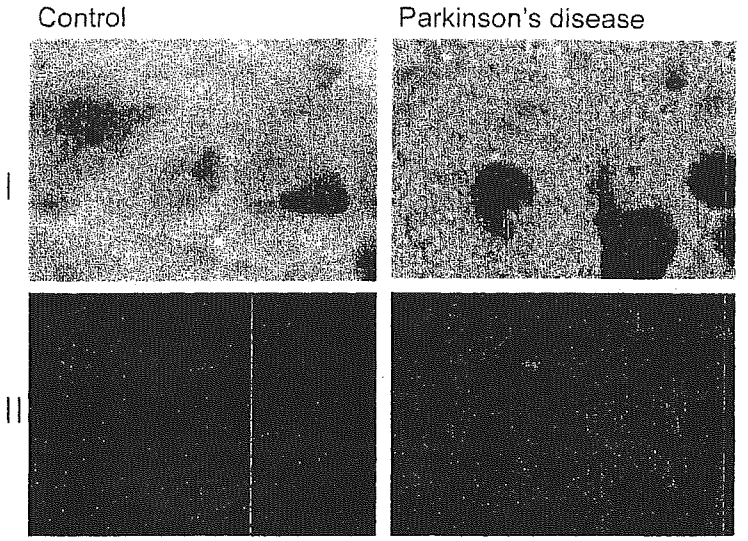

Figure 4 Occurrence of 4-HNE-modified protein in dopamine neurons in substantia nigra of the brain from the patient with Parkinson's disease. Only in the substantia nigra of the brain from the parkinsonian patient, but not from control, dopamine neurons containing neuromelanin are positively stained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

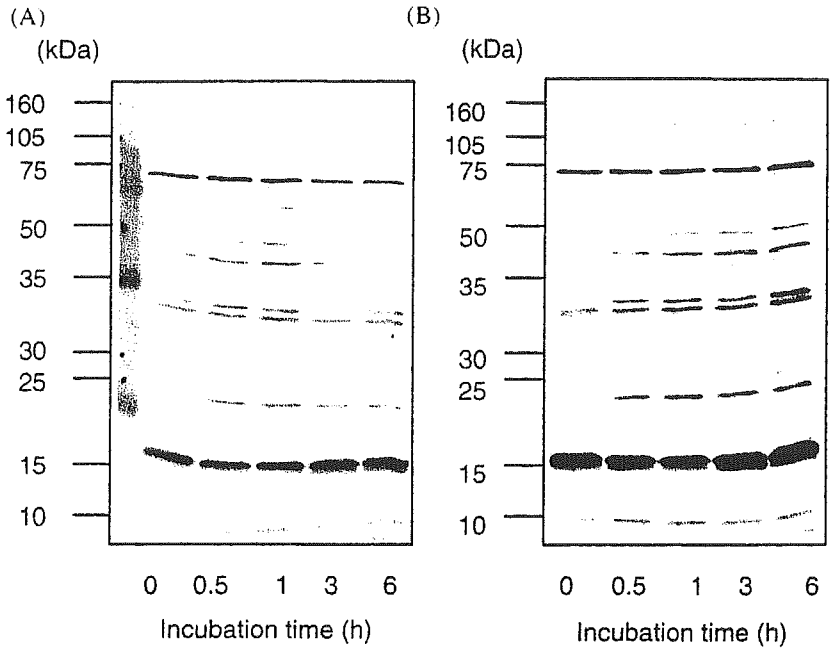

Figure 5 Formation of 3-NT protein in SH-SY5Y cells and the effects of peroxynitrite-generating SIN-1 and a proteasome inhibitor, PSI. (A) Cells were treated with 250 μM of SIN-1, then applied to the immunoblotting with antibody against 3-NT proteins. After the treatment with SIN-1, the intensity of 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

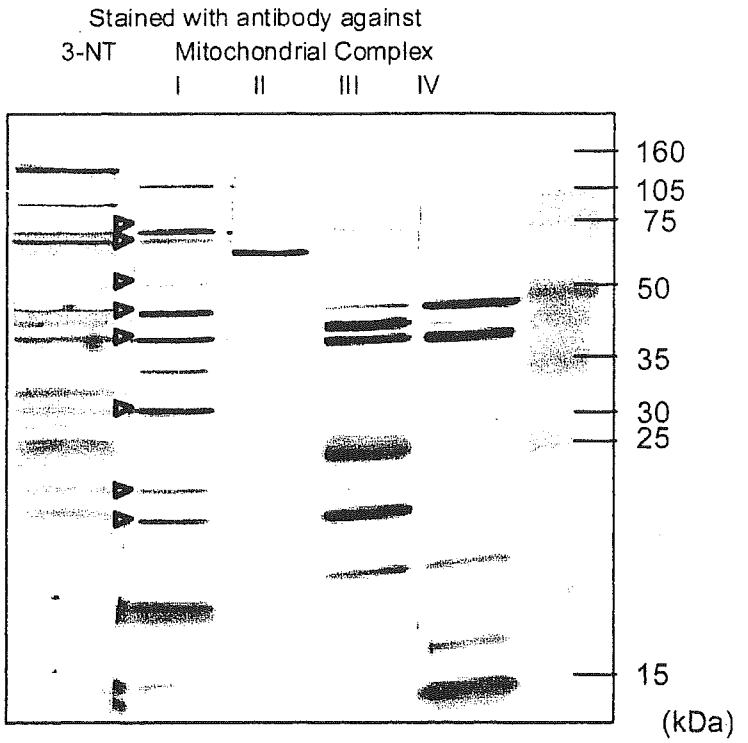

Figure 6 Detection of 3-NT proteins in mitochondria complex I–IV subunits. The cells were treated with 250 μM SIN-1 for 1 hr, and P2 fraction was isolated. The samples were subjected to SDS-PAGE and detected by the immunoblot assay using the antibody against 3-NT , or complex I (I), complex II (II), complex III (III), and complex IV (IV), respectively. Molecular markers are shown in the right column. Arrows show the protein bands positively stained with anti-3-NT and anticomplex I antibody.

the subunits of complex I, as assigned based on the staining with anticomplex I antibody (Fig. 6). These results clearly show that the preferential nitration of complex I subunits may contribute to mitochondrial dysfunction observed in the nigro-striatum of parkinsonian brain (30,31).

Treatment of $ONOO^-$-generating *N*-morpholino sydnonimine (SIN-1) induced apoptosis in the SH-SY5Y cells (32–35).

A step-wise activation of apoptotic cascade was observed: decline in $\Delta\Psi m$, activation of caspase 3, and phosphorylation of p38 mitogen-activated phosphokinase (MAP) (33). Nitric oxide and $ONOO^-$ were reported to induce apoptosis by nitration of tyrosine residues to release cytochrome *c* (36), or of cytochrome *c* itself (37). In addition, mitochondrial ATP synthesis was inhibited markedly by $ONOO^-$, as shown in Fig. 7, which may be due to its reversible binding to cytochrome oxidase or inactivation of complex I and II, and ATPase (38–41).

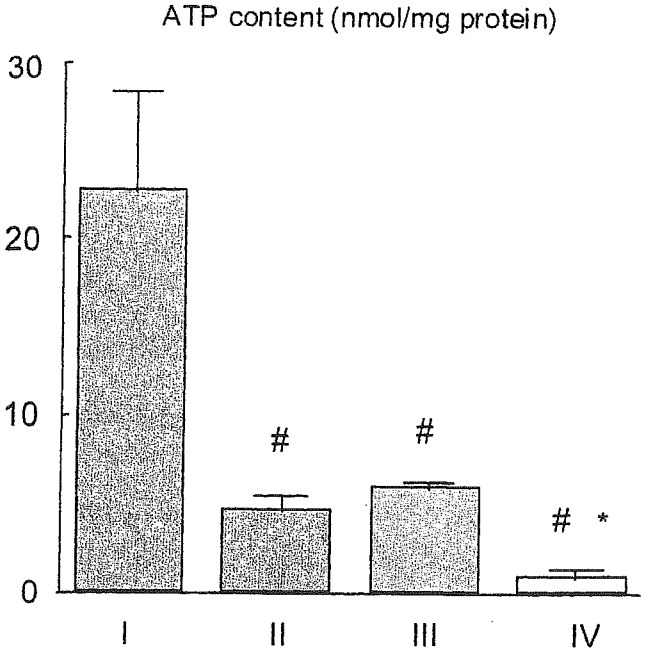

Figure 7 Effect of SIN-1 and PSI on the ATP levels in SH-SY5Y cells. The cells were incubated without or with 250 μM SIN-1 in the absence and presence of 10 μM PSI for 2 hr. The ATP content was measured by the luminofluorometric method. The column and bar represent the mean and SD of triplicate measurements of three independent experiments. I, control cells; II, cells treated with PSI alone; III, SIN-1 alone; and IV, SIN-1 and PSI. # Difference from control (I) is statistically significant ($p < 0.05$). * Difference from SIN-1 alone (III) is statistically significant ($p < 0.05$).

As shown in Fig. 5A, SIN-1 treatment increased 3-NT protein only in distinct protein bands, suggesting the preference of some proteins to $ONOO^-$ modification. In addition, increase in the amount 3-NT protein was not so significant.

III. PROTEASOME: ITS ROLE IN ACCUMULATION OF OXIDIZED PROTEINS

Insoluble intracellular protein aggregates, such as Lewy body in PD and senile plaques composed of β-amyloid in AD, are hallmarks of neurodegeneration. Although it remains unclear whether protein aggregates cause directly neuronal cell death or are the results of deteriorated cellular homeostasis in dying neurons, the analysis of the constituents of inclusion bodies may suggest the molecular mechanism leading their formation. Protein aggregation is a manifestation of disturbed cellular protein-folding homeostasis maintained by the ubiquitin–proteasome system. In LB, ubiquitin, and proteasome, subunits are major components (42,43), in addition to α-synuclein, Parkin, and ubiquitin C-terminal hydrolase-L1 (UCH-1) (44–47). They were modified with ROS–RNS as nitrated synuclein (48) and dityrosine (49).

To clarify the interactions among oxidative stress, dysfunction of the proteasome system, and formation of the inclusion body, the effects of a proteasome inhibitor, carbobenzoxy-L-isoleucyl-γ-*t*-butyl-L-alanyl-L-leucinal (PSI) were examined on the deposition of modified proteins and the cell vulnerability (34,50). PSI increased the amount of 3-NT proteins in the SH-SY5Y cells, but the number of 3-NT protein bands was almost the same as in the control (Fig. 5B). The functional deterioration of mitochondria was also enhanced by PSI, as shown by the severe reduction of ATP synthesis (Fig. 7). In addition, the number of apoptotic cells increased significantly by PSI, whereas that of necrotic cells remained almost the same (Fig. 8A). At the same time, the acrolein-modified protein increased significantly in subcellular fractions of the SH-SY5Y cells after being treated with PSI (Fig. 8B). These results clearly demonstrate that the inhibition of proteasome activity