PEROXYNITRITE INDUCES NEURONAL CELL DEATH IN AGING AND AGE-ASSOCIATED DISORDERS: A REVIEW

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ABSTRACT

Peroxynitrite produced from nitric oxide and superoxide has been proposed to cause neuronal dysfunction and cell death in aging and age-related degenerative diseases. 3-Nitrotyrosine, an oxidation product of tyrosine by peroxynitrite, was reported to increase in degenerating brains. In this paper, involvement of peroxynitrite in neuronal cell death was studied by analyses of human brains and *in vitro* experiments on cell death induced by a peroxynitrite-generating agent, SIN-1. 3-Nitrotyrosine-containing proteins were detected in lipofuscin, a typical aging-related pigment in human brains. The cytotoxicity of peroxynitrite was examined in human dopaminergic SH-SY5Y cells by use of SIN-1. SIN-1 induced apoptotic cell death in the cells, and increased the level of 3-nitrotyrosine-containing proteins. The intracellular transduction of death signal was studied in apoptosis induced by peroxynitrite. Apoptosis was induced by sequential death cascade, collapse of mitochondrial membrane potential, activation of caspases and fragmentation of nuclear DNA. In addition, phosphorylation of p38 mitogen activated phosphokinase (MAPK) was found to be associated with apoptosis by SIN-1, as shown

by inhibition of apoptotic process by SB202190, a p38 inhibitor. Involvement of peroxynitrite in the cell death is discussed in relation to neuronal degeneration in aging and age-associated diseases.

INTRODUCTION

Oxidative stress has been proposed to induce neuronal death in aging and age-associated disorders. Nitric oxide (NO) reacts with superoxide anion produces peroxynitrite (ONOO⁻), one of the most reactive radicals among reactive oxygen (ROS) and nitrogen species (RNS) (1). Peroxynitrite is unstable, but its protonated peroxynitrous acid (ONOOH) is extremely reactive (2), which generates hydroxyl radical by homolytic cleavage (3). Peroxynitrite causes oxidative damage to lipids, proteins, DNA, carbohydrates and proteins, and main targets of nitration are sulfhydryl and hydroxyl residues in amino acids, cysteine, methionine, phenylalanine and tyrosine. It inactivates the membrane function and key enzymes (see reviews, 1, 4). A product by protein nitration, 3-nitrotyrosine, is a marker for the oxidative stress induced by peroxynitrite *in vivo* (5), detected in atherosclerosis (6), amyotrophic lateral sclerosis (1), and other neurodegenerative disorders, such as Alzheimer's disease (7, 8) and Parkinson's disease (9).

Apoptosis is now considered to be a common type of cell death during embryogenesis, in neurodegenerative disorders (10, 11) and possibly also in aging (12). Oxidative stress is one of the most important stimuli to induce apoptotic cell death. Actually peroxynitrite and NO were found to induce apoptotic cell death in PC12 cells (13) and human dopaminergic neuroblastoma SH-SY5Y cells (14, 15). At present, three pathways of

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apoptotic signaling in mammalian cells have been proposed. The first pathway is initiated by withdrawal of growth factors or by neurotoxins, and regulated by Bcl-2 family of proteins. The opening of mitochondrial permeability transition pore (PTP) releases cytochrome C and other apoptosis inducing factors, which sequentially activate caspase cascade, and induce nuclear DNA fragmentation. Recently, we found that peroxynitrite opened PTP and induced the collapse of mitochondrial membrane potential, $\Delta\Psi_m$, in SH-SY5Y cells (16). The second pathway is triggered by the signal transmission from death receptors, TNF-receptor I (RI) (p55), TNF-RII (p75) and DR3 (APO3), on the cell surface, activated by tumor necrosis factor (TNF) or Fas. Association of TNF-RI with TNFR-associated death domain protein (TRADD) activates initiator caspases, caspase-8 or caspase-10, then effector caspases, caspase-3, caspase-6 and/or caspase-7 (17, 18). Binding of TNF-related proteins to death receptors triggers another apoptotic pathway. Ligation of TNF-RI or TNF-RII with TRADD or TNF receptor-associated factor 1 (TRAF1) activates mitogen activated protein kinase (MAPK) cascade (19). Recently we reported the involvement of p38 MAPK in apoptosis induced by peroxynitrite (15). However, it remains to be clarified how peroxynitrite initiates apoptotic signal transduction in the cells.

This article reports occurrence of 3-nitrotyrosine in lipofuscin, a typical pigment in aged brains, and induction of apoptotic cell death in SH-SY5Y cells by N-morpholino sydnonimine (SIN-1), which produces NO and superoxide simultaneously resulting in the formation of peroxynitrite. The death signal in response to peroxynitrite was studied in concern to the protein nitration and activation of death cascade and p38 MAPK. The results are discussed in relation to the mechanism of neuronal cell death in aging and age-related neurodegenerative diseases.

MATERIALS AND METHODS

Chemicals

SIN-1, inhibitors of nitric oxide synthase, N⁵-nitro-L-arginine methyl ester (L-NAME), N⁵-(1-iminoethyl)-L-ornithine (L-NIO), S-isopropylisothiourea hydrobromide (IPIT) and iminopiperidine (IP) and a specific scavenger of NO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (carboxy-PTIO) were purchased from Dojindo (Kumamoto, Japan). A fluorescent substrate for caspase-3 like proteases, acetyl Asp-lu-al-sp-4-methyl-coumaryl-7-amide (Ac-DEVD-MCA) and 7-amino-4-methylcoumarin (AMC) were purchased from Peptide Institute (Osaka, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Chemicon International Inc. (Temecula, CA, USA). RIPA buffer kit was obtained from Boehringer Mannheim (Mannheim, Germany); Hoechst 33342 and 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) and 2',7'-dichlorofluorescein (DCF) from Molecular Probes (Eugene, OR, USA). Antibody against 3-

nitrotyrosine was prepared as reported previously, and the specificity of this antibody and the validity for the quantitative analysis of protein-bound 3-nitrotyrosine were already reported [20]. Antibody against phosphorylated p38 was purchased from New England Biolabs (Boston, MA, USA); an inhibitor of p38, SB202190, from Calbiochem (La Jolla, CA, USA); propidium iodide (PI), Dulbecco's modified Eagle minimum essential medium (MEM), and other reagents were from Nacalai Tesque (Kyoto, Japan).

Immunohistochemical Staining of Human Brains

The brain of a 75-year-old male patient without any neurological and psychiatric disorders was obtained at autopsy, fixed in formalin and embedded in paraffin. Immunohistochemical study using anti-3-nitrotyrosine antibody was performed in a similar way to that for detection of dityrosine (21). As a blank, the samples incubated without the 1st antibody was used. The samples were stained with hematoxylin-eosin (H-E) or periodic acid-Schiff (PAS) staining to identify lipofuscin.

Generation of Peroxynitrite in SH-SY5Y Cells

For detection of ROS including peroxynitrite, H₂DCFDA was used as a fluorescent indicator (22). SH-SY5Y cells were harvested and suspended in phosphate-buffered saline (PBS) containing 2 mM sodium pyruvate, to which H₂DCFDA was added to be 50 nM. The increase in the fluorescence intensity of DCF produced from H₂DCFDA was measured at every 15 min with excitation at 504 nm and emission at 520 nm. The linearity of the DCF fluorescence to the cell protein amount and the incubation time was examined. Pyruvate-containing PBS and MEM were used for short-termed experiments to eliminate the effect of ATP supplement from glycolysis.

The effect of NO-derived radicals on DCF fluorescence was examined using nitric oxide synthase (NOS) inhibitors, L-NAME (a NOS inhibitor), L-NIO (an iNOS inhibitor), IPIT (a NOS inhibitor) or IP (an iNOS inhibitor) were used. The cells, suspended in MEM containing 2 mM sodium pyruvate, were incubated with or without 500 μ M of each inhibitor. The fluorescence of DCF produced was monitored in every 15 min. The sample incubated with DCF and each NOS inhibitor without cells was used as a blank.

Apoptosis induced by peroxynitrite

SH-SY5Y cells were cultured in 6-well flasks (1 x 10⁵ cells/well) at 37°C in 95 % atmosphere and 5 % CO₂ and the culture medium used was Cosmedium-001 (CosmoBio, Tokyo, Japan) supplemented with 5 % Nakashibetsu precolostrum newborn calf serum (Mitsubishi Kasei, Tokyo, Japan). Then, the medium was replaced with MEM and cells were incubated with various concentrations of SIN-1. Cells were cultured in 96-well flask for 24 hours and subjected to the MTT assay to determine cell viability as reported previously (15). Types of cell death were assessed by nuclear

staining with PI or Hoechst 33342, as reported previously (16, 23). Cells were incubated with anti-oxidants, superoxide dismutase (SOD) (25 unit/well) plus catalase (6 μ g/well), or carboxy-PTIO (50 μ M in the final concentration) 1 hour before addition of 1 mM SIN-1. The involvement of p38 MAPK was studied, 10 μ M or 40 μ M of SB202190, an inhibitor of p38, was added to each well in MEM 1 hour before addition of SIN-1. Then, cells were cultured for 24 hours and subjected to the MTT assay.

Immunoblotting

SH-SY5Y cells treated with 250 μ M of SIN-1 were harvested, washed twice by PBS and sonicated in the RIPA buffer and centrifuged. The protein concentration was analyzed according to Bradford (24). The cell lysate (50 μ g protein) was subjected to SDS-polyacrylamide electrophoresis, then blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) and 3-nitrotyrosine-containing proteins were detected using the antibody specific for 3-nitrotyrosine. Phosphorylated p38 was detected in cells treated with 1 mM SIN-1 by immunoblotting using antibody against phosphorylated p38.

Measurement of Enzymatic Activity of Caspase-3

The cells treated with 1 mM SIN-1 in MEM were lysed in 20 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 1 mM DTT, 5 mM EDTA, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (2.5 μ g/ml), and 1% Triton X-100 for 20 min on ice. The caspase-3 activity was assayed using a fluorescent peptide substrate, Ac-DEVD-MCA. Fluorescence at 460 nm of a fluorescent product, AMC, was measured in a spectrofluorophotometer (RF-5300PC, Shimadzu, Kyoto, Japan) with excitation at 380 nm. To examine the effects of p38 on the activity of caspase-3, the cells were pre-treated with 40 μ M of SB202190, and were harvested at 12 hours after SIN-1 treatment, and then the caspase-3 activity was measured.

RESULTS

Figure 1 shows immunohistochemical staining of the normal brain using anti-3-nitrotyrosine antibody. Lipofuscin was identified in neurons by its typical morphology (Figure 1) and also by H-E and PAS staining. Antibody against 3-nitrotyrosine positively stained lipofuscin (Figure 1A), but the sample treated without the first antibody was not stained (Figure 1B).

ROS and RNS cleave H_2DCFDA into fluorescent DCF, and this method is considered to represent the level of ROS and RNS in cells. DCF fluorescence increased in SH-SY5Y cells in a time- and dose-dependent way (Figure 2A). The cells were maintained in a medium supplemented with 2 mM pyruvate to allow only mitochondrial ATP production (25).

NOS inhibitors, L-NAME, N-NIO, IPIT and IP, reduced the production of DCF fluorescence in the SH-SY5Y cells significantly (Figure 2B).

The involvement of peroxynitrite in the induction of apoptosis is further demonstrated by use of peroxynitrite-producing SIN-1. Figure 3A shows the induction of cell death in SH-SY5Y cells after 24 hours' treatment. Using MEM without pyruvic acid, cell death was induced significantly by SIN-1 of concentrations higher than 1 mM, whereas in the presence of pyruvic acid, 250 μ M of SIN-1 induced 30 % of cell death after 18 hours. At these conditions (1 mM of SIN-1 in MEM or 250 in MEM containing 2 mM pyruvate), the type of cell death was mostly apoptotic by the morphological observation.

The specific scavengers of superoxide and NO, SOD plus catalase and carboxy-PTIO, suppressed cell death induced by SIN-1, as shown in Figure 3B.

To confirm the production of 3-nitrotyrosine by peroxynitrite, SH-SY5Y cells were incubated with 250 μ M SIN-1 in MEM containing pyruvate, which induces apoptotic cell death predominantly at this concentration. The amount of 3-nitrotyrosine-containing proteins increased after SIN-1 treatment, as shown in Figure 4. The level of modified proteins reached to a plateau 1 hour after SIN-1 treatment and declined to the basal level after 6 hours. The major 3-nitrotyrosine-containing proteins were estimated to have molecular weight of 33 kDa, 21 kDa, 15 kDa and 11 kDa. The intensity of bands representing protein-bound 3-nitrotyrosine was confirmed to linearly depend on the protein amount.

SIN-1 was found to open mitochondrial PTP, as shown by decline in $\Delta\Psi_m$ visualized by a fluorescent indicator, JC-1 (16). The $\Delta\Psi_m$ collapse was followed by activation of caspases and fragmentation of nuclear DNA. As shown in Fig. 5A, the activity of caspase-3 like proteases began to increase after 6 hours' SIN-1 incubation, and after 12 hours it increased almost 6-folds as compared with that in control, and decreased then after. The increase was statistically significant at 12 hours, compared to control ($p < 0.01$).

As another pathway to apoptosis, the involvement of p38 MAPK was studied. SIN-1 activated p38 MAPK; phosphorylated p38, detected by the immunostaining,

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

Fig. 2 Radical production in human dopaminergic neuroblastoma, SH-SY5Y cells. (A) Cells containing 0.138 (I), 0.345 (II) or 0.69 mg protein (III) were suspended in PBS containing 2 mM pyruvate and incubated with 50 nM 2,7-H₂DCF DA and the fluorescence of produced DCF was measured by a fluorospectrometer. Each circle and bar represents the mean and SD of 3 independent experiments. (B) The effects of the inhibitors of nitric oxide synthase on DCF production in the cells. Cells were incubated without (I) or with 500 μ M of L-NAME (II), L-NIO(III), IPIT (IV) or IP (V), and the fluorescence of DCF produced was quantified. Each column and bar represent mean and SD of 6 independent experiments. The samples without cells were used as blanks. *:P < 0.05 and **: p < 0.01 from the cells incubated without NOS inhibitors.

Fig. 3. Cell death of SH-SY5Y cells induced by SIN-1. A) The Cell death in SH-SY5Y cells were determined by MTT assay after incubation with SIN-1. The cells were cultured in poly-L-lysine coated flasks and treated with various concentrations of SIN-1 in MEM without pyruvic acid for 24 hours, as described in **Materials and Methods**. Each circle and bar represent mean and SD of 4 independent experiments. B) The effects of scavengers of superoxide and peroxynitrite on the SIN-1 cytotoxicity. Cells were incubated without (I) or with 1 mM of SIN-1(II), or the scavenger of superoxide, superoxide dismutase plus catalase (III), or that of nitric oxide, carboxyl-PTIO (IV) were added 1 hour before SIN-1. *: p < 0.05 compared to the cells treated with SIN-1 alone by ANOVA.

Fig. 4. Formation of protein-bound 3-nitrotyrosine in the cells treated with SIN-1. Cells were suspended in MEM containing 2 mM pyruvate and treated with 250 μ M of SIN-1, then applied to the immunoblotting, as described in Materials and Methods. After the treatment with SIN-1, the intensity of 3-nitrotyrosine-containing proteins increased. The level of 3-nitrotyrosine-modified protein reached a maximum after 1 hour of the incubation, and then decreased.

Fig. 5. Effect of p38 MAPK inhibitor on the activation of caspase-3 like proteases by SIN-1.

A). Cells were treated with 1 mM SIN-1 for the appropriate reaction time and the activity of caspase-3 like proteases was determined fluorometrically using Ac-DEVD-AMC. Values represent the means $\pm$ SEM of duplicate measurements of two independent experiments. *; The difference from the cells at zero time was statistically significant by ANOVA ($p < 0.01$).

B). After 1 hour's pretreatment with vehicle (SIN) or 40 μ M SB202190 (+SB), cells were incubated with 1 mM SIN-1 and the activity of the enzyme was determined after 12 hours. Values represent the means $\pm$ SEM of duplicate measurements of three independent experiments, and are expressed as percentage of the activity in the cells treated with SIN-1 alone. *; The difference from the cells treated with SIN-1 alone was statistically significant by ANOVA ($p < 0.05$).

increased after 30 min treatment with SIN-1, and reached to a maximum at 3 hours, as shown by Figure 6A. An inhibitor of p38 MAPK, SB202190, attenuated the cell death induced by SIN-1 in a dose-dependent manner as shown in Figure 6B. The effect of SB202190, on the activation of caspase-3 like proteases were also examined. Cells were pre-treated with the inhibitor and further incubated with SIN-1. As shown in Fig. 5B, the inhibitor suppressed the increase in caspase-3 activity, significantly ($p < 0.05$). These results suggest that MAPK is at least partially involved in the apoptotic pathway induced by SIN-1 and affects the site located in the upstream of caspase activation.

DISCUSSION

The specific marker for peroxynitrite-mediated oxidative stress, 3-nitrotyrosine, was identified in lipofuscin in the neurons. Lipofuscin is a dark pigment in the cytoplasm of postmitotic cells, which accumulates according to aging (26). Lipofuscin contains about 50% by weight of proteinaceous substances (27) and lesser amount of lipid-like materials. The distribution of lipofuscin in the human brain is uneven, being related to the levels of oxidative enzymes (28). The presence of 3-nitrotyrosine in neuronal lipofuscin is relevant with our previous observation that lipofuscin contains dityrosine, another oxidation product of proteins (21), indicates that neurons are continuously exposed to peroxynitrite-

mediated oxidative stress. In Parkinson's disease, neuromelanin, melanized lipofuscin (29), also accumulates with age especially in dopaminergic neurons, and dopamine neurons containing neuromelanin are selectively vulnerable in Parkinson's disease (30). In these neurons, dopamine is oxidized by monoamine oxidase or by autooxidation, yielding hydrogen peroxide and superoxide, and polymerized dopaquinone condensation products, as detected in neuromelanin. Hydrogen peroxide produces hydroxyl radical, and superoxide generates peroxynitrite by reaction with NO. 3-Nitrotyrosine immunoreactivity was detected in Lewy bodies, a hallmark of Parkinson's disease, suggesting that peroxynitrite may be involved in cell death of nigral dopamine neurons in Parkinson's disease (9).

In this paper, the oxidative stress induced by peroxynitrite was examined using neuroblastoma SH-SY5Y cells as a model of dopaminergic neurons *in vitro*. The results with a fluorescence indicator, 2',7'-H₂DCF, suggest that the cells are continuously producing radicals, such as hydrogen peroxide or peroxynitrite. NOS inhibitors partially but significantly reduced increase in DCF fluorescence, demonstrating that NO contributes generation of ROS and RNS.

The specific scavengers for superoxide and nitric oxide protected the cell death induced by SIN-1, respectively, suggesting that peroxynitrite itself is responsible for the induction of cell death. Even in control cells without SIN-1 treatment, proteins containing 3-nitrotyrosine were detected, suggesting again continu-

ous peroxynitrite generation in the cells. SIN-1 treatment increased the level of 3-nitrotyrosine-containing protein only transiently. It may be due to a short time of peroxynitrite generation from SIN-1 (half-time less than 60 min), and to a rapid catabolism of nitrated proteins by proteases or by nitrase (31).

The mechanism underlying induction of apoptosis by peroxynitrite was also studied in this paper. As reported in our previous paper (16), peroxynitrite was found to open PTP, as visualized by collapse in $\Delta\Psi_m$, and activate apoptotic cascade, such as activation of caspases and endonucleases (15). The further detailed mechanism to open PTP may be due to energy crisis by inhibition of mitochondrial complex I, II and III (32), which is supported by our recent finding that complex I protein was selectively nitrated by peroxynitrite (Yamamoto et al., in preparation). As another signal transduction to apoptosis, MAPK p38 was activated by SIN-1 treatment, but it requires further study to clarify how P38 MAPK activation may be related to loss of $\Delta\Psi_m$ and activation of caspase-3.

As a conclusion, our data indicate that peroxynitrite is involved in the neuronal dysfunction and death in aging and age-relating neurodegenerative disorder. Future study on protein nitration, dysfunction of enzymes, and induction of apoptosis by peroxynitrite may give a new insight in the mechanism of neuronal cell death in age and age-related disorders, and may give us new clues to protect neurons from apoptosis.

Fig. 6. Phosphorylation of p38 in SH-SY5Y cells treated with SIN-1.

A) SH-SY5Y cells were incubated with 1 mM SIN-1 for the indicated time, and applied for immunoblotting as described in Materials and Methods. Phosphorylated p38 in the cell lysate was analyzed using antibody against phosphorylated p38.

B) After 1 hour's pretreatment with 10 or 40 μM of SB202190, cells were incubated with 1 mM SIN-1 for 24 hours. The viability of the cells was assessed by MTT assay.

*: $p < 0.05$ compared to the cells treated with SIN-1 alone by ANOVA.

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