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Protective Properties of Neoechinulin A against SIN-1–Induced Neuronal Cell Death

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Peroxynitrite ($ONOO^-$) is thought to be involved in the neurodegenerative process. To screen for neuroprotective compounds against $ONOO^-$-induced cell death, we developed 96-well based assay procedures for measuring surviving cell numbers under oxidative stress caused by 3-(4-morpholinyl) sydnonimine hydrochloride (SIN-1), a generator of $ONOO^-$, and sodium *N,N*-dietyldithiocarbamate trihydrate (DDC), an inhibitor of Cu/Zn superoxide (O_2^-) dismutase. Using these procedures, we obtained a microbial metabolite that rescued primary neuronal cells from SIN-1-induced damage, but not from DDC-induced damage. By NMR analysis, the compound was identified as neoechinulin A, an antioxidant compound that suppresses lipid oxidation. We found that the compound rescues neuronal cells such as primary neuronal cells and differentiated PC12 cells from damage induced by extracellular $ONOO^-$. However, non-neuronal cells, undifferentiated PC12 cells and cells of the fibroblast cell line 3Y1 were not rescued. Neoechinulin A has scavenging, neurotrophic factor–like and anti-apoptotic activities. This compound specifically scavenges $ONOO^-$, but not O_2^- or nitric oxide (NO). Similar to known neuroprotective substances such as nerve growth factor and extracts of *Gingko biloba* leaves, neoechinulin A inhibits the SIN-1-induced activation of caspase-3–like proteases and increases NADH-dehydrogenase activity. These results suggest that neoechinulin A might be useful for protecting against neuronal cell death in neurodegenerative diseases.

Key words: free radical scavengers, neoechinulin A, neuroprotective effect, oxidative stress, peroxynitrite.

Peroxynitrite ($ONOO^-$) is produced from superoxide (O_2^-) and nitric oxide (NO) (*1*). O_2^- is highly toxic to neurons as it initiates the chain-reactive production of various reactive oxygen species (ROS) during metabolism; protection against O_2^--induced toxicity is critical for neuronal survival (*2*, *3*). NO has diverse physiological functions (*4–7*) and is toxic to neuronal cells (*8*). NO reacts with O_2^- in a diffusion-limited manner to form the more toxic oxidant $ONOO^-$ (*1*), which induces the death of PC12 cells (*9–11*) and cortical neurons (*12*). In the central nervous system, $ONOO^-$ can be generated by microglial cells activated by pro-inflammatory cytokines or β-amyloid peptide and by neurons (*13*). $ONOO^-$ is far more selective than other strong oxidant and preferentially reacts with thiols (*14*). In addition, $ONOO^-$ also reacts with tyrosine to yield 3-nitrotyrosine (*15*). Increasing levels of nitrotyrosine (*16*) are associated with degenerating neurons in the Alzheimer's disease brain, suggesting pathogenic roles for $ONOO^-$.

SIN-1 (3-(4-morpholinyl) sydnonimine hydrochloride) is a vasodilator that spontaneously releases O_2^- and NO into the medium, thereby producing $ONOO^-$ (*17*, *18*). The compound causes a concentration-dependent increase in cortical cell injury (*19*). It has been reported that neurotrophic factors such as nerve growth factor (NGF) (*20*), and free radical scavengers such as uric acid (*21*) and manganese (III) tetrakis (4-carboxyphenyl) porphyrin (Mn-TBAP) (*22*), rescue neuronal cells from SIN-1-induced damage. However, these compounds prevent oxidative damage caused by various ROS as well as $ONOO^-$ -induced damage.

Copper/zinc superoxide dismutase (Cu/Zn-SOD) is highly expressed in neurons (*23*). Thus, an SOD-inhibitor, sodium *N,N*-dietyldithiocarbamate trihydrate (DDC) elevates the amounts of intracellular O_2^- and induces oxidative damage through the chelathor of Cu^{2+} in the active site of Cu/Zn-SOD (*24*, *25*). To obtain compounds that specifically protect neuronal cells against $ONOO^-$-induced oxidative damage, we screened microbial metabolites that rescue primary neuronal cells from SIN-1-induced injury, but not from DDC-induced injury. We obtained a microbial metabolite that specifically protects against $ONOO^-$-induced cell death. In this paper, we describe the neuroprotective properties of this compound.

MATERIALS AND METHODS

Culture of Fungi and Extraction of Their Metabolite— Fungi were isolated as described by Inoue *et al*. (*26*) and incubated at room temperature for 21 d. Each culture was filtered through cheesecloth to remove the mycelia, and the components were extracted with CH_2Cl_2. The

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organic extract was evaporated *in vacuo* to yield a crude extract, which was subjected repeatedly to silica gel column chromatography using *n*-hexane–ethyl acetate as the solvent.

Cell Culture and 96-Well Based Assay Procedures—Primary neuronal cells were prepared from embryonic 17-d Wister rat brains as described by Suzumura *et al.* (*27*). In brief, the meninges were removed and the brains were dissociated by adding in Dulbecco's modified Eagle's medium (DMEM) MIXTURE F-12 HAM (Sigma Aldrich Fine Chemicals, St. Louis, MO, USA) containing 2.85 mg/ml glucose, 5 μM HEPES, 25 μg/ml insulin, 2 μM progesterone, 0.1 mM putrescine, 0.03 μM sodium selenite, 0.1 mg/ml apo-transferrin, 100 U/ml penicillin and 100 μg/ml streptomycin (DF medium).

For screening microbial metabolites, primary neuronal cells were cultured on poly-D-lysine (PDL) coated 96-well plastic plates (Becton Dickinson, NJ, USA) at an initial density of 0.7×10^5 cells/cm^2 in 2% fetal bovine serum (FBS)–DF medium for 5 d at 37°C. The cultures were treated with microbial metabolites for 24 h, and then, cell death was induced by adding 1 mM SIN-1 (Dojindo, Kumamoto Japan) or 4 μg/ml DDC (Wako, Osaka, Japan). After 24 h, and live cells were counted using a Cell Counting Kit-8 (Dojindo). The kit detects mitochondrial NADH-dehydrogenase activity in live cells by measuring the reduction of the tetrazolium monosodium salt, WST-8. This is a modified MTT assay, and it is known that the MTT assay is not influenced in the presence of various oxidants. Cell number was also measured by the CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, USA), which quantifies the amount of DNA (*28*). The chemical structures of the compounds obtained were determined by NMR analysis.

PC12 cells were incubated in 75-cm^2 tissue culture flasks in DMEM (Nissui, Tokyo, Japan) supplemented with 10% FBS, 5% horse serum (HS), 100 U/ml penicillin, and 100 μg/ml streptomycin. NGF-differentiated PC12 cells were treated with 100 ng/ml NGF for 5 d. Cells of the rat fibroblast cell line 3Y1 were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

To study the protective effects of microbial metabolites and known scavengers on PC12 cells and 3Y1 cells, cells were cultured at a initial density of 3.0×10^3 cells/cm^2 in DMEM supplemented with 10% FBS, 5% HS or DMEM containing 10% FBS 5 d at 37°C. Cell death induced by SIN-1 and cell viability were measured as described above.

All cultures were maintained at 37°C in a humidified CO_2-incubator.

Observation of Tyrosine Nitration by Immunofluorescence—Primary neuronal cells were cultured for 7 d on PDL-coated 8-well culture slides (Becton Dickinson). The cells were fixed with 4% paraformaldehyde in PBS (+) (phosphate-buffered saline containing 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+}) for 1 h, washed three times with PBS (+), and incubated with 10% FBS-PBS (+) at 4°C for 1 h. Next, they were incubated with an anti-nitrotyrosine antibody 1AE (Upstate, VA, USA) in 10% FBS-PBS (+) at 4°C for 1 h. After two washes with PBS (+), the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (H&L) (ICN Pharmaceuticals, Inc. Morgan, Irvine, CA, USA) in 10% FBS-PBS (+) for 1 h. Cell nuclei were stained with 10 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich Fine Chemicals) at 37°C for 30 min. The cells were then washed with PBS (+), and the nuclear DNA was observed under a fluorescence microscope IX70 (OLYMPUS, Tokyo, Japan).

Measurement of Scavenging Activity—We measured $ONOO^-$ and O_2^- scavenging activities using the chemiluminescent procedure described by Radi *et al.* (*29*) and Beauchamp and Fridovich (*30*), respectively. In brief, 400 mM 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) (Sigma Aldrich Fine Chemicals) was diluted with 5 mM $NaHCO_3$, pH 10.5 (Wako) in Hank's balanced salt solution (HBSS) (Invitrogen Corp, Carlsbad, CA, USA) (solution A). Test compounds were diluted with HBSS (pH 7.0) (solution B). Solutions A and B and 0.5 mM SIN-1 diluted with HBSS (pH 7) were mixed, and then $ONOO^-$ was measured using a chemiluminometer (MicroLumat LB96V; Berthold Technology, Bad Wildbad, Germany) for 20 min. O_2^- was generated by the xanthine-xanthine oxidase system following the modified method of Beauchamp and Fridovich (*30*). Specimens in Tris-HCl buffer were added to the wells of 96-well plates, each well containing 10 U/ml xanthine oxidase and 2 μM 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydriimidazo-[1,2-α]pyrazin-3-one (MCLA; Tokyo Kasei Co. Ltd, Tokyo, Japan). To generate O_2^-, 30 μM xanthine was added to each well. Chemiluminescence intensity was measured with a chemiluminometer for 10 min. NO scavenging activity was measured by the fluorometric method of Kojima *et al.* (*31*). The fluorometric detection of NO was carried out using the NO indicator (NONOate; Dojindo) and diaminofluorescein-2 (DAF-2; Daiichi Pure Chemicals Co. Ltd, Tokyo, Japan). The fluorescence from DAF-2T, the reaction product of DAF-2 with NO, was measured as the fluorescence intensity of DAF-2T using a microplate fluorescence reader (Packard Instrument Co., Meriden, USA) (Ex = 490 nm and Em = 520 nm). Mn-TBAP (Dojindo), a scavenger of $ONOO^-$ and O_2^-, and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (C-PTIO, Dojindo), a scavenger for NO, were used as controls.

Caspase Activity Assay—We measured caspase-3 activity according to Zhou *et al.* (*32*). After exposure to $ONOO^-$, cells were incubated in 50 mM Tris-HCl buffer (pH7.4) containing 4 mM DTT (Sigma Aldrich Fine Chemicals), 2 mM EDTA, 10% glycerol, 0.1% Triton X-100 and 20 μM Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarine (Sigma Aldrich Fine Chemicals), a fluorogenic substrate for caspase-3. The activity of caspase-3-like proteases was determined by measuring the fluorescence intensity of the cleaved substrate in a microplate fluorescence reader (Packard Instrument Co., Meriden, USA) (Ex = 360 nm and Em = 460 nm).

RESULTS

Screening of Microbial Metabolites That Prevent SIN-1–Induced Neuronal Cell Death—We screened two hundred microbial metabolites and obtained one compound that rescued primary neuronal cells from SIN-1-induced death. This compound was identified as neoechinulin A by NMR analysis. In the presence of SIN-1, the viability

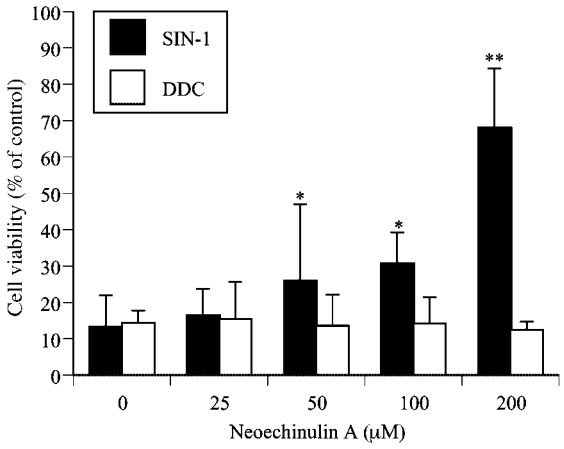

Fig. 1. **Dose-dependent effects of neoechinulin A on neuronal cells.** Primary neuronal cells were seeded at a density of 1.4×10^5 cells/cm^2. After 5 d, cells were incubated with neoechinulin A for 24 h, and treated with 1 mM SIN-1 or 4 μg/ml DDC for 24 h. Cell viability was measured using a Cell Counting Kit-8 (means ± SD, n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$.

of primary neuronal cells was less than 15%. Treatment with 200 μM neoechinulin A increased cell viability to more than 60%, while such treatment provided no protection against cell death induced by DDC (Fig. 1). This indicates that neoechinulin A protects primary neuronal cells against $ONOO^-$-induced death, but not against O_2^--induced death.

3Y1 cells, a fibroblast cell line, were not rescued from SIN-1–induced death by neoechinulin A. To determine the protective specificity of neoechinulin A, its effect on NGF-differentiated and undifferentiated PC12 cells was examined. Neoechinulin A rescued only differentiated PC12 cells (Fig. 2), suggesting that protective properties of the compound are specific to neuronal cells. Differentiated PC12 cells were also dose-dependently rescued by neoechinulin A (Fig. 3.). The results, shown in the Figures 2 and 3, were confirmed using another cell counting kit, the CYQUANT Cell proliferation kit (data not shown).

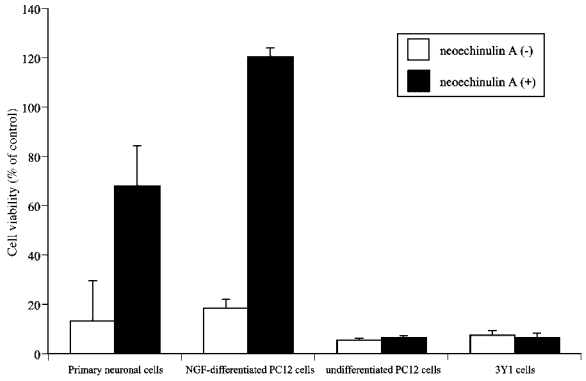

Fig. 2. **Preventive effect of neoechinulin A against SIN-1 injury.** Primary neuronal cells (1.4×10^5 cells/cm^2), PC12 cells (3.0×10^3 cells/cm^2) and 3Y1 cells (3.0×10^3 cells/cm^2) were cultured on PDL-coated 96-well plates for 5 d. After treatment with or without 200 μM neoechinulin A for 24 h, cells were cultured in the presence of 1 mM SIN-1 for 24 h. Cell viability was determined using a Cell Counting Kit-8.

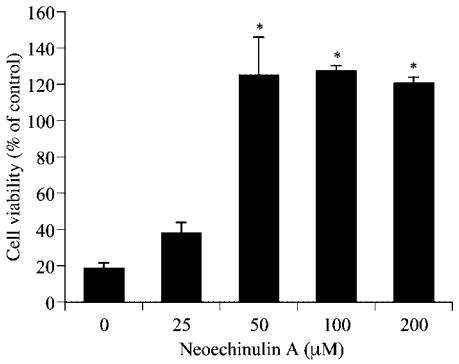

Fig. 3. **Dose-dependent effects of neoechinulin A on differentiated PC12 cells.** PC12 cells were seeded at a density of 3.0×10^3 cells/cm^2 and treated with 100 ng/ml NGF for days. Differentiated PC12 cells were incubated with neoechinulin A for 24 h, and treated with 1 mM SIN-1 for 24 h. Cell viability was measured using a Cell Counting Kit-8. (means ± SD, n = 4) $^{*}p < 0.01$.

Ability of Neoechinulin A to Scavenge ROS Produced by SIN-1—To study the scavenging activity of neoechinulin A, we examined the nitration of tyrosine residues in SIN-1–exposed cells by immunostaining with monoclonal anti-nitrotyrosine antibody 1AE. Tyrosine nitration was almost completely inhibited by neoechinulin A (Fig. 4), suggesting that neoechinulin A has $ONOO^-$ scavenging activity. This activity was confirmed by the chemiluminescence procedure. The $ONOO^-$ scavenging activity of neoechinulin A is comparable to the activities of Mn-TBAP and C-PTIO (Fig. 5A). Scavenging either O_2^- or NO also prevents the generation of $ONOO^-$ from SIN-1. Mn-TBAP and SOD scavenge about 100% and 75%, respectively of O_2^-, while neoechinulin A does not scavenge O_2^- (Fig. 5B). The fluorometric procedure using DAF-2 revealed that C-PTIO scavenges about 60% of NO, while neoechinulin A does not eliminate NO (Table 1). These results indicate that neoechinulin A specifically scavenges $ONOO^-$, but not O_2^- and NO.

Neuroprotective Effects of Neoechinulin A in SIN-1-Induced Oxidative Stress—It has been reported that various substances, such as anti-apoptotic compounds and neurotrophic factors, have neuroprotective activities (*33*, *34*). We confirmed the neuroprotective effect of the scavengers, C-PTIO and uric acid (Fig. 6A). To examine the activities of neoechinulin A other than scavenging activity, differentiated PC12 cells were pretreated with neoechinulin A, C-PTIO and uric acid for 24 h, and then, after removal of the compounds, with SIN-1 for 24 h. Under these conditions, the scavengers did not rescue differentiated PC12 cells. As shown in Fig. 6B, neoechinulin A still had a neuroprotective effect. This result suggests that neoechinulin A activities other than its scavenging activity.

Figure 3 shows that the viability of neoechinulin A–treated cells was increased by 120%. In this study, live cells were counted with a cell counting kit that measures NADH-dehydrogenase activity as described in "MATERIALS AND METHODS." Several researchers have reported that NGF and bFGF increase the mitochondrial NADH-

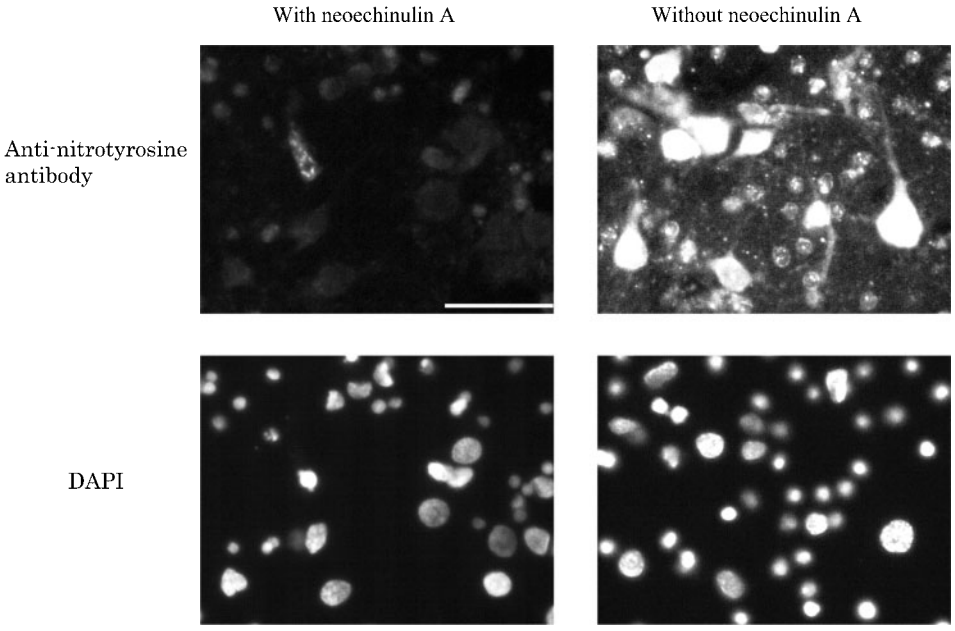

Fig. 4. **Prevention of ONOO⁻-induced tyrosine nitration by neoechinulin A.** Primary neuronal cells were incubated with 0.25 mM SIN-1 for 2 h with or without 200 μM neoechinulin A, and stained with an anti-nitrotyrosine antibody 1AE and DAPI as described in "MATERIALS AND METHODS." Bar = 50 μm

dehydrogenase activity of PC12 cells (*35*, *36*). These facts suggest that neoechinulin A activates this enzyme in PC12 cells. To examine this possibility, we investigated the effect of neoechinulin A on NADH-dehydrogenase in differentiated PC12 cells in the absence of SIN-1. NADH-dehydrogenase activity was normalized to the number of cells as measured with the assay kit that counts cell number based on quantification of genomic DNA. A significant increase in NADH-dehydrogenase activity was observed after the addition of neoechinulin A (Fig. 7).

$ONOO^-$ activates caspase-3 (*37*, *38*). Thus, we examined the effect of neoechinulin A on the SIN-1–induced activation of caspase-3-like proteases in differentiated PC12 cells. The activation was suppressed by about 65% by 200 μM neoechinulin A. On the other hand, neoechinulin A did not influence the caspase-3–ike protease activity of 3Y1 cells (Fig. 8). These results indicate that neoechinulin A has neurotrophic factor-like and anti-apoptotic activities.

DISCUSSION

Neoechinulin A is a known metabolite of *A. rubber* and *A. amstelodami* (*26*). Yagi *et al*. (*39*) reported neoechinulin A to be an antioxidant compound that suppresses lipid peroxidation in dried bonito flakes, *Katsuobushi*. However, additional properties of the compound were not investigated. In this paper, we show that neoechinulin A has scavenging, neurotrophic factor-like and anti-apoptotic activities. The results shown in Fig. 5 and Table 1 indicate that the compond scavenges only $ONOO^-$ released

Table 1. **NO generation in the presence of scavengers.**

Compounds	Generation of NO (% of control)
None	100
Neoechinulin A	100 ± 3
C-PTIO	41 ± 1
SOD	108 ± 5

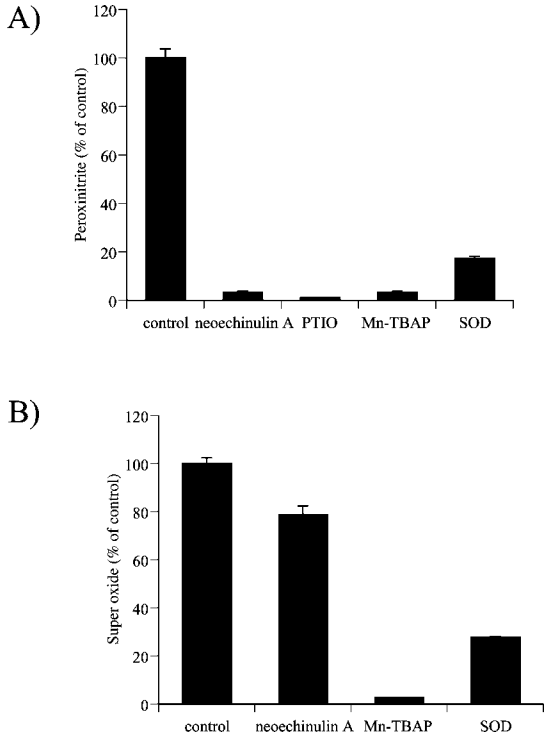

Fig. 5. **Scavenging activity of neoechinulin A.** (A) Measurement of $ONOO^-$ by luminol-dependent chemiluminescence. Luminol was diluted to 400 mM with 5 mM $NaHCO_3$ (pH 10.5) and the samples were diluted with HBSS, pH 7, prior to the measurement of chemiluminescence. Just before measurement, 0.5 mM SIN-1 diluted with HBSS, pH7, was added, and the amount of $ONOO^-$ was measured by a chemiluminometer for 20 min. (B) Measurement of O_2^- by luminol-dependent chemiluminescence. Samples in 100 μl of Tris-HC1 buffer, pH 7.4, containing 10 U/ml xanthine oxidase were incubated in a 96-well plate, and 1 μM of MCLA (50 μl) were added. Xanthine (30 μM, 50 μl) was added to generate O_2^-, and the amount of $ONOO^-$ was measured by a chemiluminometer for 20 min.

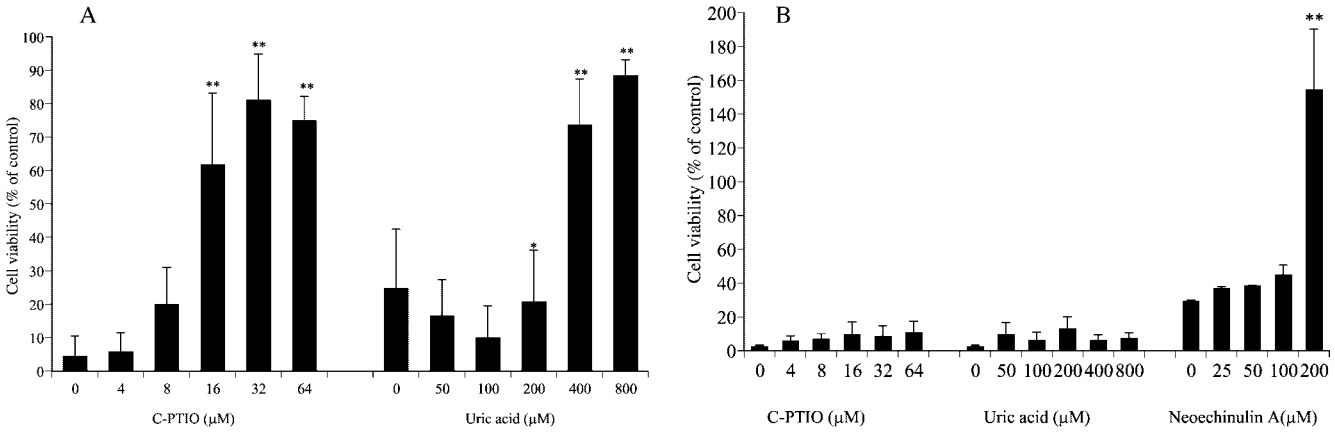

Fig. 6. **Neuroprotective effect of neoechinulin A and scavengers.** PC12 cells were seeded at a density of 3.0×10^3 cells/cm^2 and treated with 100 ng/ml NGF for 5 d. Cell viability was measured using a Cell Counting Kit-8. (A) Cells were incubated with scavengers for 24 h, and treated with 1 mM SIN-1 for 24 h. (B) Cells were incubated with neoechinulin A for 24 h. The neoechinulin A was removed and the cells were treated with 1 mM SIN-1 for 24 h. (means ± SD, $n = 4$) $^{*}p < 0.05$, $^{**}p < 0.01$.

from SIN-1. In addition, neoechinulin A specifically protects neuronal cells against SIN-1–induced cell death (Fig. 2). Thus, neoechinulin A is very useful for investigations of $ONOO^-$-induced neuronal cell death.

$ONOO^-$ is a powerful oxidant and cytotoxin whose production has been associated with conditions that result in damage to neurons. The appearance of nitrotyrosine immunoreactivity in postmortem brain from individuals with Parkinson's disease and other neurodegenerative conditions (*40–42*) also serves as indirect evidence of $ONOO^-$ production. It is well known that extracts of *Gingko biloba* leaves (EGb 761) rescue neuronal cells against ROS-induced cell death (*43*, 44). EGb 761 includes two major groups of constituents, flavonoids and terpenoids, that are involved in scavenging and antiapototic activities. It has been reported that EGb 761 increases the level of mRNA for the mtDNA-encoded subunit 1 of NADH-dehydrogenase (*45*) and suppresses the activation of caspase-3 caused by various apoptosis inducers (*46*). In the case of neoechinulin A, one compound has scavenging, neurotrophic factor-like and antiapoptotic activities. It should be noted that neoechinulin A scavenges $ONOO^-$, but not NO, because NO has various physiological functions (*4–7*). Therefore, neoechinulin A may be useful for protection against $ONOO^-$-induced neuronal cell death in neurodegenerative diseases.

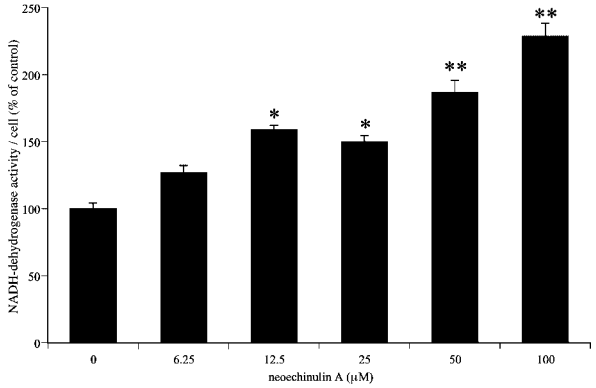

Fig. 7. **Effect of neoechinulin A on the NADH-dehydrogenase activity of PC12 cells.** PC12 cells were seeded at a density of 3.0×10^3 cells/cm^2 and treated with 100 ng/ml NGF for 5 d. The indicated concentration of neoechinulin A was added to each well and the relative NADH-dehydrogenase activity per cell was determined as described in "MATERIALS AND METHODS" (means ± SD, $n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$.

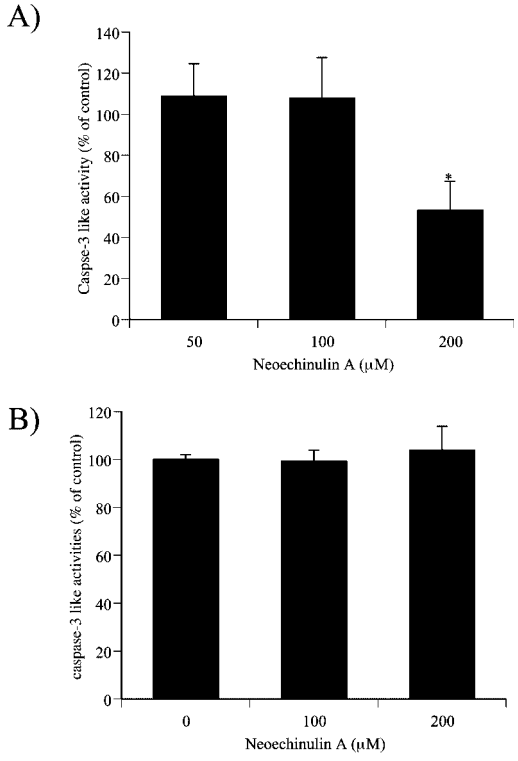

Fig. 8. **Effect of neoechinulin A on caspase-3 like protease activity.** PC12 cells (A) and 3Y1 cells (B) were seeded at a density of 3.0×10^3 cells/cm^2. PC12 cells were treated with 100 ng/ml NGF. Five days later, the cells were incubated with neoechinulin A for 24 h. After removal of the neoechinulin A, 0.5 mM SIN-1 was added. After 3 h, caspase activity was measured as described in "MATERIALS AND METHODS" (means ± SD, $n = 4$). $^{*}p < 0.01$.

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