Neoechinulin A Imparts Resistance to Acute Nitrosative Stress in PC12 Cells: A Potential Link of an Elevated Cellular Reserve Capacity for Pyridine Nucleotide Redox Turnover with Cytoprotection

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Treatment of PC12 cells with fungus-derived alkaloid neoechinulin A for more than 12 h renders the cells resistant to subsequent superoxide ($O_2^{\cdot-}$)/nitric oxide (NO) insults derived from 3-morpholinosydnonimine (SIN-1). However, the underlying mechanism(s) remains largely unclear. To elucidate the mechanism(s), we assessed the specificity of the cytoprotection afforded by neoechinulin A treatment using other cytotoxic stressors and also clarified the resulting cellular alterations, focusing on the antioxidant and metabolic enzymes systems. Neoechinulin A treatment for more than 12 h endowed PC12 cells with significant resistance to transient NO toxicity, but not persistent NO toxicity, bolus H_2O_2 toxicity, or oxidative insult from the redox cycling quinone menadione. Cellular antioxidant system profiling revealed no substantial potentiation of the activity of any antioxidant enzyme in lysate from the neoechinulin A-treated cells excluding glutathione (GSH) content, which was significantly decreased (>50%), resulting in a proportional compromise in the thiol-reducing activity of the intact cells. In addition, no differences were observed in the activity for any nicotinamide adenine dinucleotide (phosphate) reduced form (NAD(P)H)-generating enzyme, steady-state NAD(P)H/nicotinamide adenine dinucleotide (phosphate) oxidized form (NAD(P)⁺) ratios, or the levels of total NAD(P)H. Nevertheless, the neoechinulin A-treated intact cells exhibited increased NAD(P)H redox turnover when driven by extracellular tetrazolium. The structurally inactive analog preechinulin failed to protect cells against NO toxicity or induce these alterations, suggesting their link with the cytoprotective mechanism. These results suggest that neoechinulin A, despite disabling the GSH defense system, confers cytoprotection against nitrosative stresses by elevating the cellular reserve capacity for NAD(P)H generation, which could offset crippling of energy-supplying systems due to nitrosative stress.

Key words neurodegeneration; nitrosative stress; reserve capacity; glutathione; 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfonylphenyl)-2H tetrazolium

Neoechinulin A is an indole alkaloid consisting of indole, diketopiperazine, and isoprenyl moieties (Fig. 1). It has been isolated from various fungi and bacteria²⁾ (see references therein). Neoechinulin A has been reported to have antiproliferative activity toward various tumor cell lines³⁾ and proinflammatory activity in the lungs⁴⁾; however, the biological activities of this alkaloid have not been fully investigated.

Excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is associated with the pathogenesis of a range of neurodegenerative diseases including Alzheimer and Parkinson diseases.^{5,6)} Therefore, compounds that can ameliorate neuronal dysfunction resulting from oxidative/nitrosative stresses have potential as therapeutics for these diseases. Previously, we found that the treatment of differentiated PC12 cells with neoechinulin A can render the cells resistant to oxidative/nitrosative stress derived from the superoxide ($O_2^{\cdot-}$) and nitric oxide (NO) cogenerator 3-morpholinosydnonimine (SIN-1).^{2,7,8)} Subsequent studies also demonstrated that neoechinulin A can also impede the progression of PC12 cell death induced by 1-methyl-4-phenylpyridinium (MPP⁺)⁹⁾ and rotenone,¹⁰⁾ both of which are well-established Parkinson

disease-inducing neurotoxins. However, the cytoprotective mechanisms against SIN-1 and neurotoxins are probably different. Cytoprotection against SIN-1 requires a minimum of 12 h of pretreatment before SIN-1 challenge,²⁾ suggesting the involvement of the induction of cytoprotective genes, such as antioxidant enzymes, in the mechanism. By contrast, for cytoprotection against MPP⁺ or rotenone, the co-presence of

Fig. 1. Structures of Neoechinulin A and Preechinulin (A) Neoechinulin A, (B) Preechinulin

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neoechinulin A during mitochondrial toxin exposure is indispensable, suggesting that a chemical property of the alkaloid, such as antioxidant activity,^{2,8)} may also play a role.^{9,10)}

In pursuing the cytoprotective mechanisms of neoechinulin A, we have thus far revealed that the alkaloid itself has potent antioxidant activity against lipid peroxidation⁸⁾ and SIN-1-induced nitration of tyrosine residues in proteins.^{2,8)} At the cellular level in intact cells, neoechinulin A treatment for a duration exceeding 12h increases the ability of cells to reduce water-soluble tetrazolium (WST-8) under the catalysis of phenazine methosulfate, indicative of increased cellular nicotinamide adenine dinucleotide (phosphate) reduced form (NAD(P)H) production.²⁾ Moreover, the double bond at C8/C9 on the neoechinulin A molecule, which constitutes a conjugated system from the indole through diketopiperazine moieties, is a structural requirement for the cytoprotective actions and antioxidant activities of neoechinulin A.^{8,10)} Nevertheless, in-depth molecular and cellular mechanisms for the cytoprotective action of neoechinulin A remain unknown.

To elucidate the mechanism, in this study, we investigated the specificity of cytoprotection imparted by neoechinulin A treatment for 24h and potential cellular alterations associated with the treatment, focusing on key antioxidant and metabolic enzymes. We demonstrated herein that neoechinulin A preferentially affords cytoprotection against transient NO stress, but not persistent NO or oxidative stresses. Neoechinulin A treatment is associated with an elevation of the cellular reserve capacity for NAD(P)H recycling and, paradoxically, a decrease in cellular glutathione (GSH) content. Potential cytoprotective mechanisms are discussed in relation with these cellular alterations.

MATERIALS AND METHODS

Reagents Acetyl CoA, 1-chloro-2,4-dinitrobenzene (CDNB), cytochrome *c*, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), Dulbecco's modified Eagle's medium (DMEM) (041-29775), glucose-6-phosphate (G6P), glutathione (GSH), iodoacetic acid, isocitrate, nitroblue tetrazolium (NBT), oxaloacetic acid, perchloric acid, RPMI 1640 medium (189-02025), and 5-sulfosalicylic acid were purchased from Wako Pure Chemicals (Osaka, Japan). Cell Counting Kit-8, (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR-3), and 3-morpholinopyrrolidine (SIN-1) were purchased from Dojindo (Kumamoto, Japan). ATP, buthionine sulfoximine (BSO), catalase, dihydroethidium (DHE), 2-hydroxyethyl disulfide, menadione, phenazine ethosulfate, D-(−)-3-phosphoglyceric acid, 3-phosphoglyceric phosphokinase, triethanolamine, and xanthine were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Diethylenetriaminepentaacetic acid (DETAPAC), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), FAD, oxidized glutathione (GSSG), reduced and oxidized forms of nicotinamide adenine dinucleotide (NADH and NAD⁺), reduced and oxidized forms of nicotinamide adenine dinucleotide phosphate (NADPH and NADP⁺), and xanthine oxidase were purchased from Nacalai Tesque (Kyoto, Japan). Alcohol dehydrogenase, glucose-6-phosphate dehydrogenase (G6PDH), and glutathione reductase (GR) were purchased from Oriental Yeast (Tokyo, Japan). Sodium pyruvate were purchased from MP Biomedicals (Fountain Pkwy, OH, U.S.A.). Dicumarol,

and 2,6-dichloroindophenol (DCPIP) were purchased from Tokyo Kasei (Tokyo, Japan). (Z)-1-[N-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate) was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). N-Acetyl-Arg-Leu-Arg-7-amido-4-methylcoumarin was purchased from Bachem Bioscience (King of Prussia, PA, U.S.A.). Other chemicals and salts were of analytical grade. Neoechinulin A and preechinulin were synthesized as described previously.⁷⁾

Cell Treatment and Viability Assay PC12 cells, a rat pheochromocytoma cell line, were routinely maintained in DMEM containing 5% horse serum, 10% bovine calf serum, penicillin G (100 U/mL), and streptomycin (100 µg/mL) in a humidified atmosphere of 5% CO₂ at 37°C. Cell treatment and cytotoxicity assays were performed as described previously.^{8,10)} Briefly, PC12 cells were seeded into poly-L-lysine-coated 96-well plates at a density of 3000 cells/well and treated with nerve growth factor (NGF; 50 ng/mL) for 4 d in RPMI 1640 medium containing 10% FCS and antibiotics (RPMI/FCS). The differentiated cells were subsequently incubated with neoechinulin A or its vehicle (0.1% dimethyl sulfoxide (DMSO)) in RPMI/FCS for an additional 24 h. Neoechinulin A treatment (100 µmol/L) for 24 h did not affect the cell number (95±12% of control, *n*=5).

For cytotoxicity assays, the control and neoechinulin A-pretreated cells were exposed to various oxidative/nitrosative stressors in RPMI/FCS, typically for 24 h, and cell viability was evaluated by lactate dehydrogenase (LDH) assay.¹⁰⁾ In some experiments, cell viability was also evaluated using MTT assay. After treatment with SIN-1 or NOR-3, cells were incubated with fresh medium containing MTT (0.5 mg/mL) for 2 h. Formazan was dissolved in 150 µL of DMSO, and absorbance at 570 nm was measured. NGF (50 ng/mL) was included in RPMI/FCS throughout the experiments.

Measurement of Cellular Antioxidant and Metabolic Enzyme Activities PC12 cells in 100-mm dishes were NGF-differentiated and incubated with either neoechinulin A (100 µmol/L) or its vehicle (0.1% DMSO) for 24 h as described above. The cells were washed with phosphate buffered saline (PBS) and lysed on ice with 0.5% Triton X-100 in PBS. The lysates were centrifuged at 10000×*g* for 10 min at 4°C; the supernatants were collected, divided into aliquots, and stored at −80°C until use. Unless otherwise specified, enzyme assays were performed at 25°C in 96-well assay plates, and the reaction kinetics were measured using a SpectraMax 190 plate reader (Molecular Devices). Reactions were performed at 200 µL/well, and the light path length at this volume was regarded as 0.5 cm. Lysates were appropriately diluted in the lysis buffer before each assay.

Antioxidant (Enzymes) Assays Total superoxide dismutase (SOD) activity was evaluated by the cytochrome *c*/xanthine/xanthine oxidase system.¹¹⁾ Fifty microliters of lysates were mixed with 150 µL of reaction mixture in PBS to give the following final concentration for each constituent: 10 µmol/L ferricytochrome *c*, 11 mU/mL xanthine oxidase, 50 µmol/L xanthine, 10 µg/mL catalase, and 100 µmol/L DETAPAC. Changes in absorbance at 550 nm were monitored, and one unit of activity was defined as the amount of enzyme required for 50% inhibition of the rate of ferricytochrome *c* reduction. The activity was calculated using the following equation:

activity (U/mL) = $V_{\text{control}}/V_{\text{lysate}} - 1$, where V_{control} and V_{lysate} are the rates of cytochrome *c* reduction in the absence or presence of cell lysate, respectively.

GR activity was assayed by monitoring GSSG-dependent NADPH oxidation at 340 nm ($\epsilon_{340} = 6220 \text{ M}^{-1} \text{ cm}^{-1}$).¹²⁾ Lysates (100 μL) were mixed with the reaction mixture in 0.1 M NaPi (pH 7.4) to give the following final concentration for each constituent: 200 $\mu\text{mol/L}$ NADPH, 200 $\mu\text{mol/L}$ DETAPAC, and 2 mmol/L GSSG. One unit of activity was defined as 1 $\mu\text{mol/min}$ of NADPH oxidation.

Glutathione peroxidase (GPx) activity with H_2O_2 was determined by measuring GR-coupled NADPH oxidation at 340 nm.¹³⁾ Lysates (50 μL) were mixed with 100 μL of H_2O_2 in PBS and 50 μL of reaction constituents in 0.1 M NaPi (pH 7.4). The final concentration of each constituent was as follows: 200 $\mu\text{mol/L}$ H_2O_2 , 200 $\mu\text{mol/L}$ NADPH, 5 mmol/L GSH, 2 U/mL GR, 200 $\mu\text{mol/L}$ NaN_3 , and 200 $\mu\text{mol/L}$ DETAPAC. GSH-dependent NADPH oxidation was monitored as described for the GR assay. One unit was defined as 1 $\mu\text{mol/min}$ of NADPH oxidation.

NAD(P)H quinone oxidoreductase (NQO) activity was measured by the dicoumarol-inhibitable reduction of DCPIP at 600 nm ($\epsilon_{600} = 21000 \text{ M}^{-1} \text{ cm}^{-1}$).¹⁴⁾ Lysates (100 μL) were mixed with 100 μL of reaction constituents in 0.1 M NaPi (pH 7.4) to give the following final concentration for each constituent: 100 $\mu\text{mol/L}$ DCPIP, 200 $\mu\text{mol/L}$ NADPH, and 5 $\mu\text{mol/L}$ FAD with or without 10 $\mu\text{mol/L}$ dicoumarol. The dicoumarol-inhibitable DCPIP reduction rate was calculated, and one unit was defined as 1 $\mu\text{mol/min}$ of DCPIP reduction.

Thioredoxin (Trx) reductase (TrxR)-like activity was evaluated by the NADPH-dependent reduction of DTNB.¹⁵⁾ Lysates (100 μL) were mixed with 100 μL of reaction constituents in 0.1 M NaPi (pH 7.4). The final concentration of each constituent was as follows: 200 $\mu\text{mol/L}$ NADPH, 1.25 mmol/L DTNB, and 1 mmol/L DETAPAC. The NADPH-dependent change in A412 was then converted into the rate of 5-Thiobenzoic acid (TNB) generation with an extinction coefficient of 412 nm (ϵ_{412}) of $13600 \text{ M}^{-1} \text{ cm}^{-1}$. One unit was defined as the generation of 2 $\mu\text{mol/min}$ TNB with the expense of 1 $\mu\text{mol/min}$ NADPH.

Glutathione *S*-transferase (GST) activity was assessed using CDNB as a substrate.¹⁶⁾ Lysate (50 μL) was mixed with 150 μL of reaction constituents in 0.1 M NaPi to give the following final concentration for each constituent: 1 mmol/L CDNB, 2 mmol/L GSH, 0.2 mM DETAPAC, and 10% (v/v) ethanol. After subtracting the rate of non-enzymatic conjugation, the rate of net absorbance change was converted into the rate of CDNB-GSH formation by using an ϵ_{340} of $9600 \text{ M}^{-1} \text{ cm}^{-1}$. One unit of activity was defined as 1 $\mu\text{mol/min}$ of CDNB-GS formation.

S-Nitrosoglutathione (GSNO) reductase (GSNOR) activity was measured by GSNO-dependent NADH oxidation using a combined ϵ_{412} of $7060 \text{ M}^{-1} \text{ cm}^{-1}$ for GSNO and NADH.¹⁷⁾ Lysates (100 μL) were mixed with 100 μL of reaction constituents in PBS to give the following final concentration for each constituent: 100 $\mu\text{mol/L}$ GSNO, 200 $\mu\text{mol/L}$ NADH, and 500 $\mu\text{mol/L}$ DETAPAC. One unit of activity was defined as 1 $\mu\text{mol/min}$ of NADH oxidation. GSNO was synthesized from acidified nitrite and GSH as described previously.¹⁸⁾

Total GSH content was measured by the GR-coupled recycling assay.¹⁹⁾ Proteins were precipitated from cell lysates *via*

mixing with equal volumes of 10% (w/v) 5-sulfosalicylic acid, followed by centrifugation. The acid-soluble supernatants were neutralized with 33% (v/v) triethanolamine. Twenty microliters of sample were combined with reaction constituents in 0.1 M NaPi (pH 7.4) to give the following final concentration for each constituent: 200 $\mu\text{mol/L}$ NADPH, 1 mmol/L DETAPAC, 1 U/mL GR, and 600 $\mu\text{mol/L}$ DTNB. For the quantitation of GSH concentrations in culture medium, 75 μL of media were mixed directly with 125 μL of the same final concentrations of reaction constituents as above in 25 mmol/L NaPi (pH 7.4). The rates of the increase in A412 were measured and converted into GSH concentrations by using a standard curve calculated using authentic GSH.

Catalase activity was assayed on a spectrophotometer (U-3010; Hitachi, Japan). Lysates (180 μL) were mixed in 720 μL of H_2O_2 in PBS in cuvettes to give a final H_2O_2 concentration of 10 mmol/L. The rate of H_2O_2 decomposition was calculated using an ϵ_{240} of $39.4 \text{ M}^{-1} \text{ cm}^{-1}$.²⁰⁾ One unit of activity was defined as 1 $\mu\text{mol/min}$ of H_2O_2 decomposition. The authenticity of the catalase activities detected in the cell lysates was confirmed by the almost complete inhibition with 0.1 mmol/L NaN_3 .

Metabolic Enzyme Assays Hexokinase (HK) activity was measured by means of G6PDH-coupled NADPH generation.²¹⁾ Lysate (50 μL) was mixed with 150 μL of reaction constituents in 50 mmol/L triethanolamine/HCl buffer (pH 7.4) to give the following final concentration for each constituent: 1.25 mmol/L glucose, 5 mmol/L ATP, 4 mmol/L MgCl_2 , 0.5 mmol/L NADP^+ , and 1 U/mL G6PDH. The rate of ATP-dependent NADPH generation was monitored at 340 nm, and one unit of activity was defined as 1 $\mu\text{mol/min}$ of NADPH generation.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity was assessed in the gluconeogenic direction.²²⁾ Lysate (50 μL) was mixed with 150 μL of reaction constituents in 50 mmol/L triethanolamine/HCl buffer (pH 7.4) to give the following final concentration for each constituent: 1 mmol/L 3-phosphoglyceric acid, 5 mmol/L ATP, 0.2 mmol/L NADH, 3 U/mL 3-phosphoglyceric phosphokinase, 1 mmol/L DETAPAC, and 5 mmol/L MgCl_2 . The rate of NADH oxidation in the steady-state phase was used for calculation using an ϵ_{340} of $6220 \text{ M}^{-1} \text{ cm}^{-1}$. One unit of activity was defined as 1 $\mu\text{mol/min}$ of NADH oxidation.

Citrate synthase (CS) activity was measured by means of coupled DTNB reduction with CoA generated from the reaction of acetyl CoA and oxaloacetate.²³⁾ Lysate (50 μL) was mixed with reaction constituents in 150 μL of 50 mmol/L Tris-HCl (pH 7.4) to give the following final concentration for each constituent: 100 $\mu\text{mol/L}$ acetyl CoA, 200 $\mu\text{mol/L}$ DTNB, 500 $\mu\text{mol/L}$ oxaloacetate, and 250 $\mu\text{mol/L}$ DETAPAC. TNB release was monitored at 412 nm ($\epsilon_{412} = 13600 \text{ M}^{-1} \text{ cm}^{-1}$), and one unit of activity was defined as 1 $\mu\text{mol/min}$ of TNB generation.

The activities of G6PDH and 6-phosphogluconate dehydrogenase (6PGDH) were determined by the rates of NADPH generation at 340 nm.^{24,25)} Lysate (100 μL) was mixed with 100 μL of reaction constituents in PBS to give the following final concentration for each constituent: 250 $\mu\text{mol/L}$ NADP^+ , 100 $\mu\text{mol/L}$ DETAPAC, and 250 $\mu\text{mol/L}$ G6P. G6PDH activity was calculated from the initial rate of NADPH generation. Due to the accumulation of 6-P-glucono- δ -lactone, the reaction rate increased with time and became constant, where the

level of 6-P-glucono- δ -lactone reached a steady state. 6PGDH activity was calculated by subtracting the rate of maximum NADPH generation from G6PDH activity observed at the initial stage. One unit of activity was defined as 1 μ mol/min of NADPH generation.

NADP⁺-isocitrate dehydrogenase (ICDH) activity was measured by the rate of isocitrate-dependent NADPH generation at 340 nm and 37°C.²⁶⁾ Lysates (100 μ L) were mixed with 100 μ L of reaction constituents in PBS to give the following final concentration for each constituent: 500 μ mol/L NADP⁺, 200 μ mol/L DETAPAC, 2 mmol/L MgCl₂, and 2 mmol/L isocitrate. One unit of activity was defined as 1 μ mol/min of NADPH generation.

NADH- and NADPH-linked diaphorase activities were evaluated using NBT as an electron acceptor. Lysates (100 μ L) were mixed with 100 μ L of reaction constituents in 50 mmol/L Tris-HCl (pH 7.4) to give the following final concentration for each constituent: 1 mmol/L NBT, 2.5 mmol/L ethylenediamine tetraacetic acid (EDTA), and 200 μ mol/L NADH or NADPH. Lysate-dependent NBT reduction activity was expressed as the rate of the change of absorbance at 550 nm per mg of protein in the well (mAbs/mg).

Proteasome activity was measured using a fluorescent peptide substrate (Ac-Arg-Leu-Arg-7-amino-4-methylcoumarin). Lysate (100 μ L) was mixed with 100 μ L of the substrate and DETAPAC in 0.1 mol/L NaPi (pH 7.4) to give final concentrations of 10 μ mol/L and 0.1 mmol/L, respectively, and then incubated at 37°C. 7-amino-4-methylcoumarin fluorescence was measured using a fluorescence plate reader (Fluoro Count, Packard, Meriden, U.S.A.) with excitation and emission wavelengths of 380 and 460 nm, respectively, and the activity was calculated from an 7-amino-4-methylcoumarin standard curve. The addition of the proteasome inhibitor MG-132 (final concentration, 10 μ mol/L) completely inhibited the lysate-dependent 7-amino-4-methylcoumarin release, thus verifying the specificity (data not shown).

Determination of the Levels of NAD(P)H Cellular pyridine nucleotide levels were measured by the dehydrogenase-mediated pyridine nucleotide recycling-coupled MTT reduction assay.²⁷⁾ Briefly, cells were lysed in a lysis buffer (0.5% Triton-X100, 20 mmol/L nicotinamide, 20 mmol/L NaHCO₃, 100 mmol/L Na₂CO₃) and centrifuged at 10,000 $\times$ *G* for 10 min at 4°C. DNA in the supernatant was removed by a 0.45- μ m filter, and the resulting extract was divided into two aliquots. One aliquot was heated at 60°C for 30 min to destroy oxidized forms of pyridine nucleotide, and the other aliquot was kept on ice to detect total pyridine nucleotides. Heat-treated and non-treated extracts were subjected to PES-catalyzed MTT reduction assays. To detect NADP(H), NADP⁺ was recycled by G6P and G6PDH. The final concentrations in 200 μ L were as follows: 0.4 mmol/L MTT, 1.6 mmol/L phenazine ethosulfate, 0.2 mmol/L G6P, 1.6 U/mL G6PDH, 8 mg/mL bovine serum albumin (BSA), 0.8 mmol/L EDTA, and 80 mmol/L Tris-HCl (pH 8.0). To detect NAD(H), NAD⁺ was recycled by ethanol and alcohol dehydrogenase. The final concentrations in 200 μ L were as follows: 0.4 mmol/L MTT, 0.8 mmol/L phenazine ethosulfate, 12 U/mL alcohol dehydrogenase, 0.6 mmol/L ethanol, and 8 mg/mL BSA in 80 mmol/L Tris-HCl (pH 8.0). The rate of change of A570 was monitored, and each pyridine nucleotide concentration was calculated from a standard curve made using the respective

NAD(P)H concentrations. The concentration of NAD(P)⁺ was calculated by subtracting the concentration of NAD(P)H (heat-stable) from the respective concentration of total pyridine nucleotides [NAD(P)H+NAD(P)⁺]. Supplementation of a known concentration of NAD(P)H or NAD(P)⁺ into PC12 cell extract resulted in an additive increase in the levels of the respective pyridine nucleotide, confirming the specificity of the assays (data not shown).

Measurement of the Thioltransferase Activity of Cells Cellular thioltransferase activity was measured according to the procedure of Biaglow *et al.*²⁸⁾ with some modifications. Briefly, after treatment without or with neoechinulin A (100 μ mol/L) for 24 h in 12-well plates, the medium was removed, and the cells were washed with PBS. Cells were incubated with 500 μ L of 5 mmol/L 2-hydroxyethyl disulfide (HEDS) in PBS containing 10 mmol/L glucose, 1 mmol/L MgCl₂, and 1 mmol/L CaCl₂ at 37°C for 15 or 30 min. The media were collected, 2-mercaptoethanol in the media was detected with 200 μ mol/L DTNB, and the absorbance was measured at 412 nm. Reduced thiol content was calculated using GSH as a standard. The thioltransferase activity was normalized by the concentration of cell protein remaining in the well after the assay.

WST-8 Reduction Assay Cellular capacity to reduce extracellular tetrazolium was measured by using the Cell Counting Kit-8, which contains 5 mmol/L WST-8, 0.2 mmol/L 1-methyl phenazine methosulfate (mPMS), and 150 mmol/L NaCl.²⁹⁾ mPMS can diffuse across the plasma membrane, and it undergoes non-enzymatic reduction by cytosolic NAD(P)H. The reduced mPMS then diffuses out of cells and reduces extracellular WST-8 non-enzymatically. Thus, the formazan formation reflects the cellular ability to regenerate NAD(P)H.

After neoechinulin A treatment for 24 h, culture medium was replaced with a fresh medium containing 10% (v/v) kit-8 solution, and cells were incubated for the indicated times. The medium was transferred to a new 96-well assay plate, and A450 was measured on a plate reader. The values were normalized by the amount of protein in the respective wells.

Detection of Cellular Superoxide Production Cellular superoxide production was measured using the fluorescent dye dihydroethidium (DHE) under fluorescent microscopy.³⁰⁾ Cells in 8-chamber culture slide (BD Falcon 354108) were treated with neoechinulin A (100 μ mol/L) or its vehicle (0.1% DMSO) for 24 h and then incubated with DHE (10 μ M) in serum-free RPMI medium for 40 min in a CO₂ incubator. Cells were washed three times with the medium, and images were acquired from seven randomly chosen fields using an inverted epifluorescence microscope (Axiovert-200, Carl Zeiss) with constant settings. Average fluorescence intensity per cell in each field was quantitated using Image J software (NIH). To achieve a maximum DHE oxidation, antimycin A (10 μ M) was added simultaneously with DHE.

Other Assays Protein concentration was determined by a BCA method (Pierce Biotechnology, Rockford, U.S.A.) using BSA as a standard.

Statistics Data were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's test. *p* values less than 0.05 were considered significant.

RESULTS

Neoechinulin A Treatment Renders PC12 Cells Resistant to Acute NO Toxicity We previously reported that treatment of NGF-differentiated PC12 cells with neoechinulin A for a minimum period of 12h renders the cells resistant to subsequent challenge with SIN-1,²⁾ an O_2^- /NO cogenerator with a half-life of approximately 30min.³¹⁾ Although SIN-1-induced cytotoxicity in cultured cells is generally assumed to result from ONOO⁻, the reaction product of O_2^- and NO, our recent study suggested that the toxic entity of SIN-1 in PC12 cell culture is not ONOO⁻ *per se*, but is a reaction product of O_2^- /NO generated in the culture medium.³¹⁾ Thus, the system is too complicated for a mechanistic study with neoechinulin A. To gain insight into the cytoprotective mechanism of neoechinulin A, we first assessed whether neoechinulin A treatment for 24h could also confer resistance to other oxidative and/or nitrosative stressors. To this end, two NO donors with different half-lives, NOR-3 ($t_{1/2}$ =30min)³²⁾ and DETA-NONOate ($t_{1/2}$ =20h),³³⁾ were employed for NO toxicity, and bolus H_2O_2 and menadione were employed for oxidative cytotoxicity studies. The naphthoquinone menadione generates O_2^- and H_2O_2 through redox cycling reactions consuming cellular NADPH and also causes arylation of thiols in cellular proteins, thus imposing a severe compound oxidative stress on cells.¹⁴⁾

Neoechinulin A (100 μ mol/L) treatment for 24h resulted in significant resistance to subsequent challenge with SIN-1, in agreement with previous studies^{2,7,8)}; the LD₅₀ was doubled from 0.6mmol/L to 1.2mmol/L (Fig. 2A). Neoechinulin A treatment also significantly ameliorated the cytotoxicity of NO from NOR-3; the LD₅₀ increased from 120 μ mol/L to 180 μ mol/L (Fig. 2B). Cytoprotection against NO was also observed at a lower neoechinulin A concentration of 50 μ mol/L, but the extent was far less than that observed at 100 μ mol/L (data not shown). Notably, however, neoechinulin A treatment did not at all confer protection against DETA-NONOate, another NO donor with a longer half-life (Fig. 2C). Moreover, neoechinulin A also failed to show substantial protection against bolus H_2O_2 (Fig. 2D) or menadione (Fig. 2E); in fact, neoechinulin A treatment slightly potentiated the cytotoxicity of menadione. The protective effects of neoechinulin A treatment against SIN-1 and NOR-3 were also confirmed by MTT assay (Fig. 3). These results suggest that neoechinulin A preferentially confers cytoprotection against acute or transient nitrosative stresses caused by NO or NO-derived reactive species (*i.e.*, SIN-1), but not against persistent NO stress or oxidative stresses caused by agents other than NO.

Similarities of Neoechinulin A-Mediated Cytoprotection against SIN-1- and NOR-3-Induced Cytotoxicities Previous studies on the structure–activity relationship demonstrated that the C8/9 double bond of neoechinulin A, which constitutes a conjugated system from the indole to the diketopiperazine ring, is an essential structural feature for the cytoprotective action of the alkaloid against SIN-1; substitution of the double bond with a single bond (preechinulin, Fig. 1B) abolishes the cytoprotective activity.⁸⁾ To clarify whether the C8/9 double bond is also essential for the cytoprotective action against transient NO toxicity, the effect of preechinulin was addressed. As shown in Fig. 4A, the cytoprotective activity of preechinulin against NOR-3 toxicity was marginal, demonstrating that the C8/9 double is also an important structural

feature for cytoprotection against transient NO stress.

Our previous study also revealed that neoechinulin A treatment for more than 12h was required to impart cytoprotection against SIN-1 toxicity in PC12 cells.²⁾ Therefore, we next addressed the minimum period of neoechinulin A treatment that can confer cytoprotection against NOR-3 toxicity. Cells were incubated with neoechinulin A or its vehicle for 1, 3, 12, or 24h and then exposed to NOR-3 (100 μ mol/L) for 24h. Significant cytoprotection was observed when the duration of neoechinulin A treatment exceeded 12h (Fig. 4B), consistent with the case of SIN-1 toxicity. The requirement for >12h of pretreatment suggests that cellular alterations involving the induction of cytoprotective genes are possibly associated with the cytoprotective action of neoechinulin A against acute nitrosative stresses induced by SIN-1 or NOR-3.

Profiling of the Antioxidant Enzyme Activities of PC12 Cells Following Neoechinulin A Treatment To identify the cellular alterations induced by neoechinulin A treatment, we first focused on the antioxidant defense system. PC12 cells were incubated with 100 μ mol/L neoechinulin A for 24h, and the activities of key antioxidant enzymes were measured in the cell lysates.

Total SOD activity ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), which reflects mostly cytosolic CuZnSOD, was similar between the control and neoechinulin A-treated cells (Table 1). Catalase resides in peroxisome and decomposes H_2O_2 therein. Interestingly, the activity of catalase ($2H_2O_2 \rightarrow 2H_2O + O_2$) was slightly decreased by neoechinulin A treatment (Table 1). NQO reduces various quinone compounds obligatorily *via* two electrons ($Q + NADPH + H^+ \rightarrow QH_2 + NADP^+$, where *Q* represents a quinone compound), thereby preventing O_2^- formation through semiquinone radical autooxidation.¹⁴⁾ However, if hydroquinone is unstable to autooxidation, as in the case of menadione, it may act as a deleterious ROS producing enzyme.³⁴⁾ However, no difference in NQO activity was observed between the control and neoechinulin A-treated cells (Table 1).

GSH is a prime cellular antioxidant compound and is a critical co-substrate for GSH-dependent antioxidant/detoxification enzymes. Unexpectedly, however, GSH content was decreased by >50% by neoechinulin A treatment (Table 1). GPx is the cytosolic enzyme responsible for the elimination of H_2O_2 and lipid peroxides using GSH ($2GSH + LOOH \rightarrow GSSG + LOH + H_2O$, where LOOH represents a lipid peroxide). The GPx activity of cells was slightly decreased with neoechinulin A treatment, although the change was not statistically significant. GR reduces GSSG back to GSH ($GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$). GST accelerates the nucleophilic reaction of GSH with various electrophiles ($GSH + X \rightarrow GS-X$, where X is an electrophile). No differences were observed in these GSH system enzymes after neoechinulin A treatment. GSNOR, originally identified as a member of alcohol dehydrogenase class III, is responsible for the reductive breakdown of GSNO ($GSNO + NADH + H^+ \rightarrow$ glutathione sulfonamide + NAD^+).³⁵⁾ Several studies reported that GSNOR plays an important role in defense against nitrosative stresses.^{36,37)} However, no elevation in GSNOR activity was observed by neoechinulin A treatment (Table 1). In parallel with the GSH system, cells also deploy another thiol-based antioxidant system, the Trx/Trx reductase (TrxR) system. We measured the activity of TrxR [$Trx-S_2 + NADPH + H^+ \rightarrow Trx-(SH)_2 + NADP^+$] by using as an alternative substrate, DTNB.

Fig. 2. Effect of Neoechinulin A Treatment on the Cytotoxicity of Various Oxidative/Nitrosative Stresses

Differentiated PC12 cells in 96-well plates were incubated with neoechinulin A (100 μmol/L) or its vehicle (0.1% DMSO) for 24 h, followed by exposure to the indicated concentrations of SIN-1 (A), NOR-3 (B), DETA-NONOate (C), H₂O₂ (D), or menadione (E) in a fresh medium for an additional 24 h. Cell viability was assessed by LDH leakage (%) in each well. The values shown are the mean ± intra-assay deviation expressed as the S.D. from four or five wells in a representative experiment. ***p* < 0.01; ****p* < 0.001 vs. the respective control.

However, no difference was observed between the control and neoechinulin A-treated cells (Table 1). Overall, these results demonstrate that neoechinulin A paradoxically and selectively decreased GSH content without substantially affecting the activities of other antioxidant enzymes.

Characteristics of GSH Depletion Induced by Neoechinulin A To gain insight into the mechanism and biological significance of GSH depletion induced by neoechinulin A treatment, the time course for the decrease in cellular GSH content was measured (Fig. 5A). After adding fresh medium, GSH content in the control cells increased gradually.

However, in the neoechinulin A-treated cells, GSH was severely depleted by 8 h, and its levels remained decreased up to 16 h, after which its levels slightly increased over the next 8 h. In fact, an approximately 50% decrease in GSH levels could be observed as early as 3 h (data not shown). Interestingly, when cells were incubated with preechinulin, GSH levels were almost unchanged throughout the 24-h incubation period. Because the acid-soluble extract used for the GSH recycling assay may contain a metabolite of neoechinulin A or preechinulin that can interfere with the enzymatic GSH assay and thereby artifactually decrease GSH readouts, a known

Fig. 3. Evaluation of the Cytoprotective Effect of Neoechinulin A against Cytotoxicities of SIN-1 and NOR-3

Control and neoechinulin A-treated cells were exposed to the indicated concentrations of SIN-1 (A) or NOR-3 (B) as in Fig. 2. Cell viability was measured by the MTT assay. The values shown are the mean $\pm$ intra-assay deviation expressed as the S.D. from four or five wells in a representative experiment. * p <0.05; ** p <0.01; *** p <0.001 vs. the respective control.

concentration of GSH was added to the acid-soluble extract from neoechinulin A- or preechinulin-treated cells, which were then subjected to the GSH recycling assay. Essentially no interference effect was observed for either extract (data not

Fig. 4. Effects of Preechinulin and the Treatment Time of Neoechinulin A on the Cytotoxicity of NOR-3

(A) Comparison of the effects of preechinulin and neoechinulin A on NOR-3 cytotoxicity. Cells were pretreated in the same manner described in the legend to Fig. 2 and then exposed to NOR-3 (100 μ mol/L). (B) Effect of neoechinulin A treatment time on the acquisition of resistance to NO cytotoxicity. Cells were treated as described in (A). In both (A) and (B), LDH leakage (%) was measured 24 h after NOR-3 treatment. The values shown are the mean $\pm$ intra-assay deviation expressed as the S.D. from four or five wells in a representative experiment. *** p <0.001 vs. the respective control.

shown), thus verifying the results. Thus, neoechinulin A did

Table 1. Effect of Neoechinulin A Treatment for 24h on the Activities of Antioxidant Enzymes and GSH Content in PC12 Cells

Antioxidant enzyme or GSH	Unit	NeoA (-)	NeoA (+)	%	<i>p</i>
Superoxide dismutase	U/mg	21.6 $\pm$ 1.4	22.2 $\pm$ 1.8	102	ns
Catalase	U/mg	2.04 $\pm$ 0.05	1.75 $\pm$ 0.14	86	*
NAD(P)H quinone oxidoreductase 1 (NQO 1)	mU/mg	9.7 $\pm$ 2.1	9.2 $\pm$ 1.7	95	ns
Glutathione (GSH)	nmol/mg	18.7 $\pm$ 0.5	8.4 $\pm$ 1.0	45	***
Glutathione reductase (GR)	mU/mg	62.7 $\pm$ 2.4	60.3 $\pm$ 2.8	97	ns
Glutathione <i>S</i> -transferase (GST)	mU/mg	11.2 $\pm$ 1.2	11.0 $\pm$ 0.0	98	ns
Glutathione peroxidase (GPx)	mU/mg	20.4 $\pm$ 0.8	17.5 $\pm$ 1.5	86	ns
GSNP reductase (GS-NOR)	mU/mg	19.5 $\pm$ 2.0	20.7 $\pm$ 2.1	106	ns
Thioredoxin reductase-like (TrxR-like)	mU/mg	16.5 $\pm$ 1.0	15.5 $\pm$ 1.5	94	ns

NGF-differentiated PC12 cells were treated with neoechinulin A (100 μ mol/L) or its vehicle (0.1% DMSO) for 24h. Cells were lysed using Triton X-100, and the GSH content and antioxidant enzyme activities of the Triton X-100-soluble fraction were measured. Values are presented as the mean $\pm$ intra-assay deviation expressed as the S.D. from three cultures. Each data set shown is one representative result from two independent experiments. * p <0.05; *** p <0.001 vs. control.

Fig. 5. Effects of Neoechinulin A and Preechinulin Treatments on Cellular GSH Content, Disulfide Reductase Activity, and Response to SIN-1

(A) Time course of the change in cellular GSH content. Cells in 6-well plates were incubated with neoechinulin A or preechinulin (each 100 μmol/L) or their vehicle (0.1% DMSO) for the indicated times, and GSH content in the cells was measured. Values are presented as the mean ± intra-assay deviation expressed as the S.D. from three wells in a representative experiment. (B) Time course of GSH accumulation in the medium. In a separate experiment, GSH concentrations in the culture medium after incubation with or without neoechinulin A (100 μmol/L) were measured. (C) Changes in cellular disulfide reductase activity by neoechinulin A treatment. Cells in 12-well plates were treated with neoechinulin A (100 μmol/L) or its vehicle (0.1% DMSO) for 24 h. The medium was removed, and the cells were washed with PBS and incubated with HDES (μmol/L) in PBS containing glucose, Ca²⁺, and Mg²⁺ for the indicated times. The levels of 2-mercaptoethanol in the medium were measured by the DTNB assay. The values are presented as the mean ± intra-assay deviation expressed as the range of two wells in a representative experiment. (D) Effect of neoechinulin A treatment on SIN-1-induced depletion of cellular GSH. Cells in 6-well plates were incubated with or without neoechinulin A (100 μmol/L) for 24 h (noted as "pretreatment") and then incubated with or without SIN-1 (1 mmol/L) in a fresh medium for an additional 2 h. Cytosolic GSH content was measured as described above. Values are presented as the mean ± intra-assay deviation expressed as the SD of three wells in a representative experiment. For (A), (B), and (D), **p* < 0.05; ***p* < 0.01; ****p* < 0.001 vs. the respective control. In (D), comparisons with other groups are also shown.

cause a decrease in cellular GSH content in PC12 cells.

It is documented that cells continuously export GSH and increase the efflux rate of GSH when exposed to certain compounds such as polyphenols³⁸⁾ and diphenyleneiodonium.³⁹⁾ To investigate whether the decrease in GSH content induced by neoechinulin A treatment was due to an increase in the rate of GSH efflux, we measured the level of extracellular GSH (Fig. 5B). After adding fresh medium, the GSH concentration in the medium increased gradually over the 20-h incubation period. However, no difference was observed between the control and neoechinulin A-treated cells.

We next examined whether the decrease in GSH content in the neoechinulin A-treated cells was reflected by the cellular redox phenotype. Reduction of exogenous 2-hydroxyethyl disulfide to 2-mercaptoethanol by live cells has been demonstrated to be catalyzed by thioltransferase glutaredoxin and significantly dependent on the substrate of the enzyme, GSH.²⁸⁾ 2-hydroxyethyl disulfide reductase activity in the neoechinulin A-treated cells was also decreased to 50% of that in the control cells (Fig. 5C), suggesting that the decrease in the levels of cellular GSH induced by neoechinulin A leads to decreased disulfide reductase activity in the cells.

Exposure to NO or SIN-1 has been demonstrated to result in GSH depletion in a range of cells *via* various mechanisms.^{40–43)} We next examined whether the neoechinulin A-treated cells undergo further GSH depletion in response to SIN-1. After treatment with neoechinulin A for 24 h, cells were challenged with SIN-1 for 2 h, and cellular GSH content was measured (Fig. 5D). Cellular GSH levels were decreased to approximately 20% of those of untreated cells after neoechinulin A treatment. Without subsequent SIN-1 treatment, GSH levels recovered to 50% of the original levels within the next 2 h. By contrast, exposure to SIN-1 for 2 h caused a severe GSH depletion in the neoechinulin A-treated cells and in the control cells. Thus, neoechinulin A-treated cells cannot prevent further depletion of residual GSH under acute nitrosative stress conditions.

Profiling of Metabolic Enzyme Activities of PC12 Cells Following Neoechinulin A Treatment We next investigated cellular alterations induced by neoechinulin A treatment for 24 h, focusing on metabolic pathways responsible for supplying NADPH for antioxidant systems and ATP production. Phosphorylation of glucose by HK is the first step of glucose catabolism (glucose + ATP → G6P + ADP). The product G6P

then undergoes oxidative breakdown reactions in either the pentose phosphate pathway (PPP) or the glycolytic pathway followed by the Krebs cycle pathway. HK activity was not different between the control and neoechinulin A-treated cells (Table 2).

The NADPH level is a critical factor for the functions of an array of cellular antioxidant enzymes. From G6P as an initial substrate, NADPH is produced by successive reactions catalyzed by two PPP enzymes, namely, the rate-limiting enzyme G6PDH ($G6P + NADP^+ + H^+ \rightarrow 6\text{-P-glucono-}\delta\text{-lactone} + NADPH$) and the second enzyme 6PGDH ($6\text{-P-glucono-}\delta\text{-lactone} + NADP^+ + H^+ \rightarrow 6\text{-P-gluconate} + NADPH$). NADPH may also be supplied from the Krebs cycle by the action of ICDH.²⁶⁾ No differences were observed in the activities of any of these NADPH-producing enzyme in response to neoechinulin A treatment (Table 2). We then measured the activities of two glycolytic enzymes responsible for NADH metabolism: LDH (pyruvate + $NADH + H^+ \rightarrow$ lactate) and GAPDH (glyceraldehyde 3-P + $NAD^+ + Pi \rightarrow$ D-glycerate 1,3-BP + $NADH + H^+$). There was also no difference in the activity of either enzyme between the control and neoechinulin A-treated cells (Table 2). CS (acetyl CoA + oxaloacetate $\rightarrow$ citrate + CoA) is a Krebs cycle enzyme, and its activity is generally reflected by the number of the mitochondria in a cell.⁴⁴⁾ No difference was observed for CS activity between the control and neoechinulin A-treated cells (Table 2).

We also measured proteasome activity; however, no difference was observed between the control and neoechinulin A-treated cells (Table 3). It is known that cells have various enzymes that can catalyze the reduction of NBT to its formazan using either NADH or NADPH (so-called diaphorase activity). A part of the activity with the latter substrate has been attributed to neuronal nitric oxide synthase (nNOS).⁴⁵⁾ In PC12 cells, diaphorase activity with NADPH was 2-fold higher than that with NADH (Table 3). However, there was no difference in either NADH- or NADPH-linked diaphorase activity in lysates from the control and neoechinulin A-treated cells. Thus,

overall, neoechinulin A treatment did not affect the activities of metabolic enzymes.

Neoechinulin A Treatment Elevates the Apparent Cellular Capacity to Generate NAD(P)H The phenazine-mediated reduction of extracellular WST-8 assay is an index of cellular capacity to generate NAD(P)H,²⁹⁾ and it is widely used for live cell counting in viability and proliferation assays. Previous studies revealed that neoechinulin A treatment elevates the WST-8-reducing capacity of PC12 cells and that a correlation exists between a potentiation of WST-8-reducing activity and an increase in resistance to SIN-1-induced cytotoxicity with several neoechinulin A analogs.²⁾ However, it remained unclear whether preechinulin can also elevate this activity. Therefore, we compared the cellular WST-8-reducing activity of neoechinulin A- and preechinulin-treated cells (Fig. 6A). After incubation with WST and phenazine, WST-8 formazan levels increased linearly with time in the control cell cultures. Consistent with previous findings,²⁾ the formazan formation rate was approximately 40% higher in the neoechinulin A-treated cells than in the control cells. By contrast, there was no increase in the formazan formation rate in cells treated with preechinulin. These results further support that the WST-8-reducing activity is linked with the cytoprotective mechanism of neoechinulin A against acute nitrosative stresses and that the presence of the C8/9 double bond is responsible for the alterations. When O_2 -dependent NADH oxidation was facilitated by the uncoupler CCCP, WST-8 reduction rates were significantly decreased to almost the same level in control and neoechinulin A-treated cells (Fig. 6A, inset graph), confirming the involvement of NADH in WST-8 reduction.

To investigate whether the elevated WST-8-reducing activity in the neoechinulin A-treated cells resulted from an increase in the reduced form of either pyridine nucleotide, the levels of NADH and NADPH and their redox states were analyzed (Fig. 6B). However, the total pools of NADH or NADPH as well as the reduced-to-oxidized ratio were not substantially different between the control and neoechinulin A-treated cells. Because various tetrazoliums including WST-1, a close

Table 2. Effect of Neoechinulin A Treatment for 24h on the Activities of Metabolic Enzymes in PC12 Cells

Metabolic enzyme	Unit	NeoA (–)	Neo (+)	%	<i>p</i>
Hexokinase (HK)	mU/mg	23.1 $\pm$ 1.8	22.9 $\pm$ 2.0	99	ns
Glucose-6-phosphate dehydrogenase (G6PDH)	mU/mg	52.6 $\pm$ 3.3	52.3 $\pm$ 1.4	99	ns
6-Phosphogluconate dehydrogenase (6PGDH)	mU/mg	12.8 $\pm$ 0.4	12.3 $\pm$ 0.5	96	ns
NADPH-isocitrate dehydrogenase (ICDH)	mU/mg	55.5 $\pm$ 1.9	57.2 $\pm$ 2.3	103	ns
Lactate dehydrogenase (LDH)	U/mg	4.4 $\pm$ 0.5	4.4 $\pm$ 0.5	100	ns
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	mU/mg	78.6 $\pm$ 6.7	85.7 $\pm$ 5.0	109	ns
Citrate synthase	mU/mg	127 $\pm$ 2	127 $\pm$ 2	100	ns

Using the same cell lysates described for Table 1, metabolic enzyme activities were measured. Values are presented as the mean $\pm$ intra-assay deviation expressed as the S.D. from three cultures. Each data set shown is one representative result from two independent experiments.

Table 3. Effect of Neoechinulin A Treatment for 24h on the Activities of Proteasome and Other Enzymes in PC12 Cells

Others	Unit	NeoA (–)	NeoA (+)	%	<i>p</i>
Proteasome	mU/mg	2.36 $\pm$ 0.09	2.20 $\pm$ 0.04	93	ns
NADH-diaphorase	mAbs/mg	50.7 $\pm$ 1.4	51.8 $\pm$ 5.6	102	ns
NADPH-diaphorase	mAbs/mg	94.9 $\pm$ 4.3	100.0 $\pm$ 8.7	105	ns

Using the same cell lysates in Table 1, the trypsin-like activity of proteasomes and diaphorase activities were measured. Values are presented as the mean $\pm$ intra-assay deviation expressed as the SD from three cultures. Each data set shown is one representative result from two independent experiments.

analogue of WST-8, can be used to detect O_2^- ,⁴⁶⁾ it may be possible that an increase in WST-8 reduction in neoechinulin A-treated cells may be due to an increase in O_2^- production by the cells. Therefore, we compared cellular O_2^- production between control and neoechinulin A-treated cells. As shown in Fig. 6C, neoechinulin A treatment did not increase basal O_2^- production or antimycin A-stimulated mitochondrial O_2^- production, thus ruling out the possibility of the elevation of O_2^- production in neoechinulin A-treated cells. Taken together, these results suggest that neoechinulin A-treated cells can enhance NAD(P)H recycling activity only when measured in intact cells.

DISCUSSION

In this study, we demonstrated that in PC12 cells, neoechinulin A afforded cytoprotection against acute NO stress induced by NOR-3 in addition to compound nitrosative stresses induced by SIN-1, but not, essentially, against persistent NO stress or oxidative stresses involving entities other than NO (Fig. 2). There are similarities regarding the cytoprotective mechanism of neoechinulin A between NOR-3- and SIN-1-induced stresses. The half-lives of SIN-1 and NOR-3 are both relatively short (*ca.* 30 min).^{31,32)} Preechinulin, which does not confer cytoprotection against SIN-1-induced toxicity,⁸⁾ also failed to confer protection against NO-induced toxicity (Fig. 4A). To provide the cells with cytoprotection against acute NO-induced toxicity, it is necessary to incubate the cells for more than 12 h with neoechinulin A (Fig. 4B), consistent with the case of SIN-1-induced toxicity.²⁾ These results suggest that the specificity of cytoprotection afforded by neoechinulin A is limited to transient nitrosative stresses, and therefore, the cytoprotective mechanism for SIN-1 and NOR-3 toxicities is probably dependent on the same cellular alterations induced by this alkaloid.

Profiling of cellular alterations revealed that substantial differences were observed only for GSH depletion and elevation of cellular WST-8-reducing activity between control and neoechinulin A-treated cells (Tables 1–3, Fig. 6A). Importantly, preechinulin treatment caused neither severe GSH depletion (Fig. 5A) nor potentiation of WST-8-reducing activity (Fig. 6A), thus suggesting a relationship between these two alterations and the cytoprotective mechanism. Moreover, regarding WST-8-reducing activity, we previously found within a series of neoechinulin A analogs a correlation between an elevation in WST-8-reducing activity and resistance to SIN-1-induced toxicity.²⁾ Thus, elevation in WST-8-reducing capacity in particular could deserve in-depth consideration in relation to the cytoprotective mechanism of neoechinulin A treatment.

Neoechinulin A Treatment Enhances Cellular Reserve Capacity for NAD(P)H Redox Turnover, Which May Be Responsible for the Cytoprotective Mechanism In a wide variety of cells including neuronal cells, excessive NO has been demonstrated to inhibit oxygen (O_2) consumption by mitochondria, initially through competition with O_2 for binding to cytochrome *c* oxidase (complex IV)⁴⁷⁾ and later by inflicting irreversible damage to the electron transport chain enzymes.^{47,48)} NO and reactive species derived from SIN-1 have also been demonstrated to impair key metabolic enzymes such as GAPDH and ICDH.^{26,49,50)} Consistent with the paradigm,

Fig. 6. Effects of Neoechinulin A and Preechinulin Treatments on the Tetrazolium-Reducing Activity of Cells and NAD(P)H Status

(A) Tetrazolium-reducing activity of intact cells. Cells in 96-well plates were incubated with neoechinulin A or preechinulin (each 100 μ M/L) or their vehicle (0.1% DMSO) for 24 h and then incubated with WST-8 along with 1-methoxy phenazine methosulfate in a fresh medium. Formazan levels were measured at 450 nm and normalized by the concentration of protein. Values are presented as the mean $\pm$ intra-assay deviation expressed as the S.D. of five wells in a representative experiment. ** p < 0.01; *** p < 0.001 vs. the respective control. The inset graph shows the effect of the uncoupler CCCP on WST-8 reduction. CCCP (10 μ M/L) was added in conjunction with WST-8. Values are presented as the mean $\pm$ intra-assay deviation expressed as the S.D. of five wells in a representative experiment. ** p < 0.01; *** p < 0.001 vs. respective vehicle-treated cells. ## p < 0.01 vs. vehicle-treated control. (B) NAD(P)H status of cells. After treatment without (0.1% DMSO) or with neoechinulin A (100 μ M/L) in 6-well plates, cellular pyridine nucleotide levels were quantitated by means of an enzyme-mediated NAD(P)H recycling-coupled MTT reduction assay. Values are presented as the mean $\pm$ intra-assay deviation expressed as the S.D. of three wells in a representative experiment. * p < 0.05 vs. respective control (C) Intracellular superoxide generation. Cells in 8-chamber slide were treated with neoechinulin A (100 μ M/L) or its vehicle (0.1% DMSO) for 24 h and then incubated with DHE (10 μ M/L) for 40 min in serum-free medium in the absence or presence of antimycin A (10 μ M/L). Images were acquired from randomly chosen seven fields, and average fluorescence intensity was calculated. Values are presented as the mean $\pm$ intra-assay deviation expressed as the SD of seven fields in a representative experiment. *** p < 0.001 vs. antimycin A (-) in respective cells.

a previous study in PC12 cells demonstrated that treatment with NOR-3 resulted in inactivation of complex IV.⁵¹⁾ We also observed that SIN-1 treatment inactivated complexes IV and V in PC12 cells (data not shown). Glial cells are reported to withstand nitrosative stresses by shifting ATP production from oxidative phosphorylation to glycolysis, whereas neuronal cells cannot shift ATP production due to lack of this metabolic shifting machinery.⁵²⁾ Taken together, damage to energy-producing systems including mitochondria and consequent energy failure,^{42,53)} could partly be responsible for the cytotoxicity of acute nitrosative stresses in our system as well. One mechanism by which neoechinulin A-treated cells can withstand acute nitrosative stresses is, therefore, the elevation of the cellular reserve capacity for NAD(P)H redox turnover, as represented by the elevated WST-8-reducing capacity.

Reduction of extracellular WST-8 *via* intermediation of the membrane-permeable electron carrier mPMS, depends on the concentration of the reduced forms of either pyridine nucleotide. The elevation of cellular WST-8-reducing capacity on neoechinulin A treatment suggests that the cells have higher concentrations of NAD(P)H. Nevertheless, neither the total concentration of NAD(H) or NADP(H) nor the ratios of their reduced-to-oxidized forms changed on neoechinulin A treatment (Fig. 6B). Moreover, none of the enzymes in PPP, the glycolytic pathway, or the Krebs cycle (Table 2) or even NAD(P)H-diaphorase activity (Table 3), was upregulated in the lysate from neoechinulin A-treated cells. These discrepant results can be reconciled if we assume that neoechinulin A-treated cells have gained the ability to increase the regeneration rate of NAD(P)H from NAD(P)⁺ to a greater extent than control cells, only when NAD(P)H is being consumed for reduction of WST-8. In other words, neoechinulin A treatment can potentiate oxidant-driven NAD(P)H redox turnover rates (reserve capacity). In the absence of the oxidant WST-8, that is, in the resting state, NAD(P)⁺/NAD(P)H steady-state ratios could remain at a certain value irrespective of such elevated turnover capacity, as the results demonstrated (Fig. 6B). The same mechanism could become operative under nitrosative stresses. If a portion of a certain enzyme in NAD(P)H-producing pathways is inactivated by nitrosative stresses, an increase in the efficiency of the pathway as a whole induced by neoechinulin A treatment can compensate for the otherwise decreased production efficiency, and thereby sustain sufficient NAD(P)H production. Currently, it is technically difficult to delineate the pyridine nucleotide for which its redox turnover reserve capacity was elevated by neoechinulin A treatment, although in cell-free system analysis, WST-8 reduction with NADH was approximately 30% faster than that with NADPH (data not shown).

Analogous situations have been reported for the cases of cellular respiratory reserve (or spare) capacity, which is defined as an increase in the O₂ consumption rate induced by uncouplers (*i.e.*, CCCP).^{54–56)} In this case, NADH oxidation is driven by O₂ *via* intermediation of the electron transport chain enzymes, in contrast to WST-8 with the intermediacy of mPMS, and the NADH supply is at least one factor determining the magnitude of the capacity.⁵⁴⁾ It has been demonstrated that various types of cells can withstand various stressors unless the reserve capacity collapses.^{44,55,56)} Forced oxidation of NADH by the presence of CCCP attenuated WST-8 reduction in the control and neoechinulin A-treated cells to the same

level (Fig. 6A, inset graph) suggests that NADH turnover is potentiated by neoechinulin A treatment.

Although the molecular mechanism for elevation of the reserve capacity for NAD(P)H turnover induced by neoechinulin A is not clear at present, one possible mechanism is that neoechinulin A treatment changes the intracellular localizations of some metabolic enzymes involved in NAD(P)H redox turnover. It has been reported that several enzymes of PPP and the glycolytic pathway are associated with mitochondria,⁵⁷⁾ scaffold proteins, and cytoskeletal proteins,^{58–60)} and their interaction can be altered in response to certain treatments.⁶⁰⁾ Cell lysis disrupts the interaction of enzymes, and therefore, activity of a component enzyme in the lysates displays no difference in the presence or absence of treatment. Thus, although the molecular details are not clear at present, elevation of the reserve capacity for NAD(P)H redox turnover induced by neoechinulin A treatment could compensate the damaged metabolic pathways resulting from nitrosative stresses, thereby affording resistance. Further studies are needed to clarify the molecular mechanism for this alteration.

Possibility of GSH Depletion as a Mechanism for Cytoprotection by Neoechinulin A Neoechinulin A treatment was associated with a selective decrease in GSH content (Table 1, Fig. 5A). This GSH decrease is unlikely due to increased efflux into the extracellular milieu (Fig. 5B). Although neoechinulin A, but not preechinulin, has a potential Michael acceptor site at C8, our cell-free study revealed that incubation of neoechinulin A with excess GSH overnight in the pH range of 7 to 9 did not result in adduct formation, as revealed by HPLC analysis (data not shown). Therefore, Michael adduct formation is unlikely to be the mechanism responsible for GSH depletion, although we could not completely rule out this possibility inside the cells because of the presence of GST. Thus, GST-mediated adduct formation, inhibition of its synthesis, and increase in their breakdown are remaining possibilities, which we need to be addressed in future research.

Whether GSH depletion plays a role in the cytoprotective mechanism of neoechinulin A is not definitive at present. However, it is well established that GSH is a pivotal cellular antioxidant against oxidative stresses and nitrosative stresses.^{36,37,61)} This paradigm is also applicable to our PC12 cells; reduction of cellular GSH content by buthionine sulfoxide (BSO), a specific inhibitor of γ -glutamyl-cysteine ligase, rendered PC12 cells significantly susceptible to SIN-1-mediated cytotoxicity³¹⁾ and to NOR-3-mediated toxicity (data not shown). Thus, it is possible to assume that, with respect to GSH content, neoechinulin A treatment severely disables the cellular GSH antioxidant defense system, indicating that other biological effects imparted by neoechinulin A must compensate for the functional decline. The elevation of the reserve capacity for NAD(P)H redox turnover by neoechinulin A treatment may have contributed to maintaining a proper GSH redox potential ([GSH]³⁾/[GSSG] ratio), in spite of a significant decrease in the total GSH pool, thereby ameliorating an otherwise weakened GSH antioxidant system. It is clear that a decrease in GSH content and an elevation of the reserve capacity for NAD(P)H turnover is not mechanistically linked. That is, while treatment with neoechinulin A or BSO for 4 h resulted in a decrease of GSH by 50% or 30%, respectively, neither treatment affected WST-8 reduction rate at this time point (data not shown).

It is interesting to note that neoechinulin A has growth-inhibitory activity in various tumor cell lines.³⁾ Tumor cells usually have defective mitochondria, which produce more ROS than normal cells.⁶²⁾ However, they also have increased GSH content, which is suggested to contribute to the high proliferation rate and apoptosis-resistant phenotype of the tumor cells.⁶³⁾ The present study implies that perturbation of the GSH redox status through GSH depletion by neoechinulin A may be in part responsible for the antitumor activity of this alkaloid.

CONCLUSION

Neoechinulin A can also afford cytoprotection against acute NO stress but not against persistent NO stress or oxidative stresses involving entities other than NO. This cytoprotection is associated with an elevation of the cellular reserve capacity for NAD(P)H redox turnover and, paradoxically, a decrease in cellular GSH content. The increased NAD(P)H production reserve capacity could compensate for the crippled metabolic systems under nitrosative stress conditions, thereby affording cytoprotection. All of these biological effects of neoechinulin A are unprecedented, and further clarification could have far-reaching effects on various biomedical fields including bioenergetics, stress responses, and neurodegeneration.

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