suggesting the "catalytic" function of NM. NADPH increased SH release by NM, but did not affect SH content in control, suggesting that NM recycling by NADPH enhances the deglutathionylation in a similar way as GRX.

The mechanism underlying induction of apoptosis by NM requires further studies to be fully elucidated. NM activated "intrinsic" apoptotic pathway, but Bcl-2 overexpression did not prevent cell death, even though Bcl-2 protects the cells against apoptosis induced by dopamine (Fig. 3 D) and a dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol (Maruyama *et al.* 2000, Akao *et al.* 2002). NM may activate inner membrane anion channel through the altered redox state depending on the GSH/GSSG ratio (Aon *et al.*, 2007), or the modification of vital SH in mitochondria. Diazenedicarboxylic acid bis 5*N*,*N*-dimethylamide (diamide) induced mitochondrial permeability transition (mPT) by modifying thiol of Cysteine-57 in adenine nucleotide translocator (ANT) localized on the matrix site at the cyclosporine-binding site (Costantini *et al.* 2000). Modification of Cysteine-57 in ANT with NO could prevent mPT, maybe by protection of the vital SH against further cytotoxic modification. EGCG, a NO scavenger, and SOD prevented the NM-induced apoptosis and increase in

mitochondrial SH, suggesting the involvement of NO, superoxide and peroxynitrite. In addition, iron released from NM may enhance ROS-RNS production, as shown by the protection of DFX. NM may remove protective GSH or cysteine from mixed disulfide bonds in mPT pores and expose vital SH to subsequent modification by ROS-RNS, resulting in induction of mPT and apoptosis, which overcomes the protection by Bcl-2.

As a conclusion, this paper clearly presents data that NM induces apoptosis in SH-SY5Y cells by means of the protein component. The mechanism is clarified as that NM deglutathionylates specified PrS-SG in mitochondrial complex I, dissociates the higher structure of mitochondria, causes the dysfunctions, and finally activates apoptosis signaling. These effects of NM on the redox state were the most manifest in isolated mitochondria, where GSH is not produced *in situ* and the level is mainly regulated by GSH recycling system composed of NADPH-dependent reductase. These situations in isolated mitochondrial may reproduce those in the substantia nigra of aged and parkinsonian brains, where the redox state tends to more oxidizing condition with reduced GSH levels. In normal condition the compensative antioxidant capacity might prevent the activation of death process by NM, even though it accumulates in the

Comment [mn1]:

substantia nigra at the concentrations of 2-4 μg/mg wet weight, almost the same levels as used in this paper, 10-25 μg/ml of reaction mixture. Further studies will clarify the more detailed role of NM in the malignant cycles between oxidative stress, mitochondrial dysfunction cell death of dopamine neurons in PD and aging. In addition, a quite new strategy may be found to prevent or delay the cell death itself by stabilization of mitochondrial redox state and *S*-glutathionylation.

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Table 1. SH contents in NM, Protease K treated NM, DAM and Cysteinyl- DAM

Melanin	SH content (nmol/mg melanin)
Neuromelanin	2.42 ± 0.80
Protease-K treated neuromelanin	0.57 ± 0.16
Dopamine-melanin	Not detected
Cysteinyl-dopamine melanin	3.77 ± 0.19

SH contents in 3 NM and 2 P-K NM samples from different brains were measured fluorometrically by use of Measure-iT™ assay kit. The values are mean ± S.D. of quadruplicate measurements of each sample.

Table 2. Total SH, GSH and GSSG contents, and GSSG/GSH ratio in the cells, subcellular fractions and isolated mitochondria after treated with NM and DAM

Fraction	Total SH (nmol/mg protein)	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSSG/GSH x 100
Cells: Control	11.9 ± 0.2	6.90 ± 0.54	0.36 ± 0.02	5.22
Treated with NM	14.2 ± 0.7*	9.61 ± 0.36*	0.88 ± 0.02*	9.16
DAM	10.2 ± 0.4	6.77 ± 0.21	0.99 ± 0.02*	14.62
Cytosol: Control	13.9 ± 1.6	10.2 ± 1.5	0.018 ± 0.003	0.18
Treated with NM	15.1 ± 1.6	12.9 ± 1.9	0.019 ± 0.001	0.15
DAM	13.4 ± 1.7	12.0 ± 1.1	0.015 ± 0.006	0.13
Mitochondria:				
Control	9.1 ± 0.1	11. 6 ± 0.2	0.55 ± 0.19	4.74
Treated with NM	11.1 ± 0.3*	19.9 ± 1.2*	1.66 ± 0.18*	8.34
DAM	7.9 ± 0.5*	13.3 ± 0.54	1.54 ± 0.11*	11.58

Isolated mitochondria:				
Control	11.7 ± 1.3	6.13 ± 0.32	0.36 ± 0.13	5.87
Treated with NM	17.1 ± 1.7*	8.54 ± 0.19*	0.88 ± 0.07*	10.30
DAM	6. 9 ± 0.2*	5.78± 0.59	0.99 ± 0.12*	17.13

The total SH contents were measured fluorometrically by use of Measure-iT™ Thiol Assay kit. GSH and GSSG were quantified using the enzymatic recycling method. The values are mean and SD of quadruplicate measurements of three experiments. *, Difference from control, $p < 0.05$.

SH-SY5Y cells were treated without or with 10 µg/ml NM or DAM for 2 h at 37°C, then subjected to subcellular fractionation according to Muyderman *et al.* (2004). The precipitated mitochondrial fraction was treated with the extraction medium for GSH/GSSG and analyzed for GSH and GSSG. Isolated mitochondria were treated with 10 µg/ml NM or DAM for 2 h at 37°C, and the total SH was measured fluorometrically, then the rest was precipitated by centrifugation, treated as above for GSH-GSSG assay.

Legends for Figures

Figure 1. Apoptosis induced by neuromelanin

A. Morphological observation of NM cytotoxicity. SH-SY5Y cells were treated without (**I**, control) or with 10 μg/ml of NM (**II**), DAM (**III**) and 100 μM dopamine (**IV**) for 16 h. The cells were observed by phase contrast, or after staining with Hoechst 33342 for apoptotic cells and with PI for dead cells.

B. FACS analyses of apoptosis. The wild ells were incubated without (**I**, control) with 25 μg/ml NM (**II**) or DAM (**III**) for 16 h, gathered, stained with PI with 1% Triton X-100, and subjected to FACS. The cells with a lower DNA content showing less PI staining than G_1 peak were defined to be apoptotic.

C. Quantitative analyses of apoptotic cell death by FACS. The wild and Bcl-2 cells were treated with 5, 10 or 25 μg/ml of NM or DAM for 16 h and were subjected to FACS. The column and bar represent the mean and SD of five experiments. * $p < 0.01$ from control.

Figure 2. Effects of protein component and melanin classes on cell viability.

SH-SY5Y cells were cultured in a 6-well tissue culture flask and treated without (**I**), or with 10 μg/ml NM (**II**), P-K NM (**III**), DAM (**IV**) or Cys-DAM (**V**) for 16 h at 37°C.

A: The number of live cells. The cells were gathered, washed with PBS and the live cells were quantitatively measured after staining with calcein. The number of live cells was expressed as percent of control, and the column and bar represent the mean and SD of quadruplicate measurements of three experiments. *, Difference from control, $p <$ 0.01.

B: Morphological observation of cells. The cells were observed by fluoromicroscopy after staining with Hoechst 33342. The cells were also treated with 100 μM dopamine (**Dopamine**).

Figure 3. Activation of apoptotic cascade by NM.

A and **B:** Reduction of $\Delta\Psi m$ by NM in mitochondria isolated from the wild (**A**) and Bcl-2 cells (**B**). **I** and **II**, NM (25 and 10 μg/ml) was incubated with mitochondria without or with 1 mM GSH (**III**, **IV**), respectively at 37C for 3 h. $\Delta\Psi m$ was measured by FACS after staining with $DiOC_6(3)$.

C: Release of cytochrome c into cytosol. After treated with NM (25 μg/ml) for 6 h, cytosol fraction was prepared from SH-SY5Y cells and subjected to Western blot analysis for cytochrome c (Cyt. c). **I**, Control. **II, III, IV** and **V**, cells were treated with NM for 1, 2, 4 and 6 h. β-Action was used as control.

D: Caspase 3 activity in the wild and Bcl-2 cells after treated for 16 h at 37°C. **I**, Control. **II, III** and **IV**, cells treated with 10 μg/ml NM and DAM, and 100 μM dopamine, respectively. Caspase 3 activity was measured fluorometrically using Ac-DEVD-MCA as a substrate. The column and bar represent the mean and SD of triplicate measurements of 4 experiments. *, Difference from control, $p < 0.01$.

Figure 4. Effects of melanin on SH levels in mitochondria.

A: Effects of NM on the mitochondrial SH contents. Mitochondria prepared from the wild (filled circle) and Bcl-2 cells (hollow circle) were incubated with NM (25 μg/ml) at 37°C, for 0.5, 1, 2 and 3 h. Mitochondria were treated without NM also in a similar way (filled and hollow square for mitochondria from wild and Bcl-2 cells, respectively). SH levels were quantified fluorometrically with the Thiol Assay Kit. The values were

represented as % of SH levels at 0 time. The point and bar represent the mean and SD of quadruplicate measurements.

B: Effects of DAM and dopamine on SH levels in mitochondria prepared from the wild cells. Mitochondria were incubated with DAM (25 μg/ml) (triangle) and dopamine (100 μM) (square). Control, circle.

C: Effects of melanin species on mitochondrial SH levels. Mitochondria were treated without (**I**, control) or with 25 μg/ml NM (**II**), P-K NM (**III**) and DAM (**IV**) for 2 h at 37°C. SH residues were quantified by the fluorometric assay with Thiol Assay Kit. The column and bar represent the mean and SD of quadruplicate measurements of 2 experiments. *, Difference from control, $p < 0.05$.

D: GSH was quantitatively measured by HPLC. Mitochondria were treated without (**I**, control) or with 25 μg/ml NM (**II**), P-K NM (**III**) and DAM (**IV**) for 2 h at 37°C. The column and bar represent the mean and SD of triplicate measurements of 2 experiments. *, Difference from control, $p < 0.05$.

Figure 5. Effects of NM and DAM on SH levels in mitochondria and subcellular