LEGENDS FOR FIGURE

Fig 1. DHA is generally neuroprotective, but potentially neurotoxic when the oxidative stress is increased.

Under the oxidative stress, DHA may produce toxic lipid peroxide and produce toxic protein adducts. Polyphenol and other food-derived antioxidant might prevent the toxicity of DHA by reducing the oxidative stress.

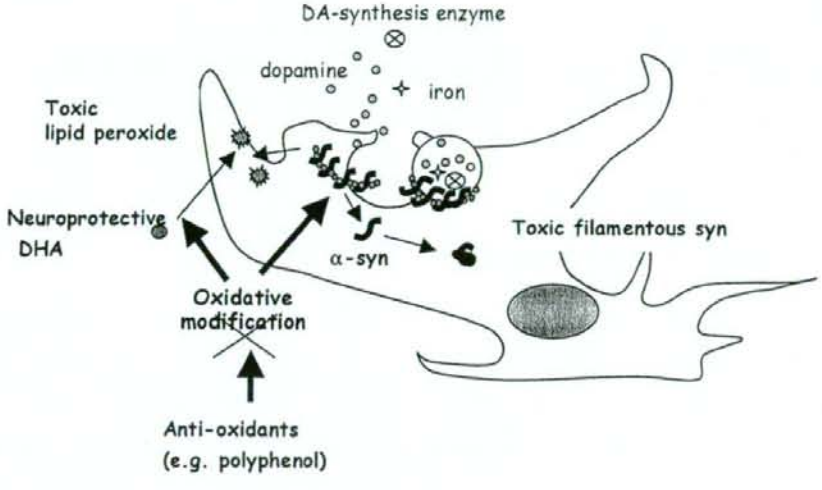
DA-synthesis enzyme
dopamine
iron
Toxic
lipid peroxide
Neuroprotective
DHA
α-syn
Toxic filamentous syn
Oxidative
modification
Anti-oxidants
(e.g. polyphenol)

Fig.1

Neuromelanin selectively induces apoptosis in dopaminergic SH-SY5Y cells by deglutathionylation in mitochondria: Involvement of the protein and melanin component

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Abbreviations used: Cys-DAM, Cysteinyl dopamine melanin; DAM, dopamine-melanin; DFX, deferoxamine mesylate; EGCG, (-)-epigallocatechin gallate; NM, neuromelanin; PD, Parkinson's disease; P-K NM, protease K treated neuromelanin; PrS-SG, *S*-glutathionylated protein

Abstract

Parkinson's disease is characterized by selective depletion of nigral dopamine neurons containing neuromelanin, suggesting the involvement of neuromelanin (NM) in the pathogenesis. This paper reports induction of apoptosis by NM in SH-SY5Y cells, whereas protease K-treated NM, synthesized dopamine- and cysteinyl-dopamine-melanin showed much less cytotoxicity. Cell death was mediated by mitochondria-mediated apoptotic pathway, namely collapse of mitochondrial membrane potential, release of cytochrome c and activation of caspase 3, but Bcl-2 overexpression did not suppress apoptosis. NM increased sulfhydryl content in mitochondria, and a major part of it was identified as GSH, whereas dopamine-melanin significantly reduced sulfhydryl levels. Western blot analysis for protein-bound GSH demonstrated that only NM reduced *S*-glutathionylated proteins in mitochondria and dissociated macromolecular structure of complex I. Reactive oxygen and nitrogen species were required for the deglutathionylation by NM, which antioxidants reduced significantly with prevention of apoptosis. These results suggest that NM may be related to cell death of dopamine neurons in Parkinson's disease and aging through regulation of mitochondrial redox state

and *S*-glutathionylation, for which NM-associated protein is absolutely required. The novel function of NM is discussed in relation to the pathogenesis of Parkinson's disease.

Keywords: neuromelanin, mitochondria, Redox status, Parkinson's disease, apoptosis, *S*-glutathionylation.

Running title: Neuromelanin causes apoptosis by modifying SH state

Neuromelanin (NM) is a pigment localized in the catecholaminergic neurons in the substantia nigra, locus coeruleus and other brainstem nuclei. In Parkinson's disease (PD), dopaminergic and noradrenergic neurons are preferentially affected (Hirsch *et al.* 1988), and the presence of NM in most of these neurons suggests that NM may be involved in the cell vulnerability (Kastner *et al.* 1992). NM is known to appear after 2-3 years of life and increase with age to a level of 2.3-3.7 mg/g wet weight of the substantia nigra pars compacta at 50-90 years of age (Zecca *et al.* 2001). A major NM composition is melanin, which is synthesized from quinones and semiquinones, products of autoxidation of dopamine, noradrenalin and L-DOPA, and accounts for about 11-13% of NM (Wakamatsu *et al.* 2003). The melanin composition is a mixture of two melanin classes, eumelanin and pheomelanin in the ratio of 1-3 to 1 (Odh *et al.* 1994). Eumelanin is black, insoluble, and composed of indole monomers derived from oxidized catechol derivatives. Pheomelanin contains about 10% sulfur, and is brownish red, alkaline soluble and produced from 5-*S*-cysteinyl-dopamine and -DOPA derived by conjugation of dopamine quinone with GSH or cysteine (Odh *et al.* 1994). Recently, studies on the surface oxidation potential of NM suggest a spherical architecture of the

pheomelanin core with eumelanin surface (Bush *et al.* 2006). In addition, protein, lipids and trace metals are detected in NM, but it has never been clarified whether these components are integrated in a complex structure of NM, or only associated to melanin in NM granules. NM contains protein components of about 15% of NM mass (Gerlach *et al.* 1995; Zecca *et al.* 2000). Proteomics of NM granules identified about 70 proteins occurring commonly in human brain tissues, mainly lysosomal proteins, suggesting non-selective protein binding to dopamine quinone (Tribl *et al.* 2005). In parkinsonian brains, α-synuclein, a component of Lewy body was reported to bind to NM or NM granules, but not in control brain (Fasano *et al.* 2003; Halliday *et al.* 2005). Solid-state NMR studies indicate the presence of glycidic and aliphatic components attributed to lipid materials, which corresponds to 20% of NM weight and identified as C_{14}-C_{18} fatty acids and dolichol, (Zecca *et al.* 2000; Fedorow *et al.* 2005). NM contains also inorganic components, iron, copper and zinc, as being about 1.5% of NM weight (Zecca *et al.* 1994). The high iron content in NM increases vulnerability of NM-containing dopamine neurons through the production of reactive oxygen and nitrogen species (ROS, RNS) (Ben-Shachar *et al.* 1991) and of cytokines and nitric oxide in microglia (Wilms *et*

al. 2003). Recently NM was found to inhibit the ubiquitin-proteasome system through increased ROS-RNS production, suggesting the involvement of NM in the accumulation of modified protein in the dopamine neurons (Shamoto-Nagai *et al.* 2004, 2006). However, NM may be also protective within dopaminergic cells by scavenging free radical species and binding toxic compounds. Human NM, but not synthesized melanin, scavenges hydroxyl radicals produced by the Fenton reaction (Li *et al.* 2005). NM binds and sequesters redox-active trace metals, dopaminergic neurotoxins, such as MPP^+ (D'Amato *et al.*, 1986) and 1,2(*N*)-dimethyl-6,7-dihydroxy-isoquinolinium ion (Naoi *et al.* 1994), and cytotoxic dopamine quinine (Youdim *et al.* 1994).

Human NM and synthetic melanin from dopamine (dopamine melanin, DAM) or L-DOPA, induced cell death in rat pheochromocytome PC12 cells (Offen *et al.* 1997) and primary mesencephalic culture cells (Nguyen *et al.* 2002). NM and DAM are phagocytosed into neuronal SK-N-SH, but not to glial U373 cells, and melanin is localized in the cytoplasm (Li *et al.* 2005). DAM-induced cell death in PC12 and SK-N-SH cells was reported to be apoptosis-like, but the detailed mechanism behind the cytotoxicity remains to be elucidated. In addition, the involvement of the NM protein

and melanin species in the cytotoxicity has been scarcely studied.

In PD, the reduction of GSH with corresponding increase of GSSG was confirmed in the substantia nigra (Riederer *et al.* 1989). GSH plays a major role in the cellular defense against oxidative stress by direct scavenging ROS-RNS, and GSH depletion initiates cell death by reduced compensation of oxidative stress. On the contrary, depletion of GSH protected cells against Fas-mediated cell death (Musallam *et al.* 2002), and *N*-acetyl-L-cysteine and GSH monoester enhanced hypoxia-induced apoptosis (Qanungo *et al.* 2004). The cytotoxic effects of GSH may be due to requirement of reducing conditions to activate caspase 3, an apoptosis executor, by caspase 8 (Hentze *et al.* 2002), and to suppress nuclear factor (NF)-κB transactivation (Qanungo *et al.* 2004). Recently, modification of protein thiols by ROS-RNS is gathering attention for its role in cellular dysfunction *via* reversible formation of mixed disulfides between the protein thiol and sulhydryl (SH) residue of GSH (PrS-SG) (Maher 2006). This posttranslational modification of protein, referred as *S*-glutathionylation, regulates the essential cellular functions, such as energy synthesis, signal transduction pathway and transcriptional activation. (Schafer and Buettner 2001). Considering that NM contains SH groups, it

should be clarified whether NM can affect *S*-glutathionylation in mitochondria and regulate the redox status, which might be involved in the selective death cascade of NM-containing dopamine neurons.

In this paper, the cytotoxicity of NM was examined in human dopaminergic neuroblastoma SH-SY5Y cells using NM prepared from human substantia nigra. To examine the roles of the protein and melanin composition in the cytotoxicity, NM was treated with proteinase K (P-K NM), and eumelanin and pheomelanin were synthesized by oxidation of dopamine in the absence (dopamine melanin, DAM) or presence of L-cysteine (cysteinyl-dopamine melanin, Cys-DAM). Wild and transfection-enforced Bcl-2 overexpressed SH-SY5Y cells (Bcl-2 cells) (Akao *et al.* 2002) and mitochondria prepared from them were used to clarify the apoptotic process. The effects of NM on intracellular redox and SH states were studied to clarify the relation of *S*-glutathionylation to cell death process. The involvement of NM in the pathogenesis of PD is discussed, where increased oxidative stress, mitochondrial dysfunction and induction of apoptosis are proposed to induce selective degeneration of dopamine neurons containing NM.

Materials and methods

Materials

3,3'-Dihexyloxcarbocyanide iodide [$DiOC_6(3)$], LIVE/DEAD viability/cytotoxicity assay kit for mammalian cells and Measure-iT™ thiol assay kit were purchased from Molecular Probes (Eugene, OR, USA); superoxide dismutase (SOD), deferoxamine mesylate (DFX), (-)-epigallocatechin gallate (EGCG) were from Sigma (St. Louis, MO, USA). A substrate for caspase 3, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid α-(4-methyl-coumaryl-7-amide) (Ac-DEVD-MCA), was obtained from Peptide Institute (Osaka, Japan). Catalase from bovine liver, Dulbecco minimum essential medium (DMEM) and other reagents were from Wako (Kyoto, Japan). SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented by 5% fetal calf serum in 95% air and 5% CO_2. Bcl-2 was overexpressed in SH-SY5Y cells (Bcl-2 cells) by transfection as reported previously (Akao *et al.* 2002).

Preparation of NM and DAM

Brains from neurologically normal adult individuals were provided from the Austro-German Brain Bank in Würzburg. The Ethics Committee of the University Clinics of Würzburg approved the use of post-mortem human brain tissue. NM was isolated from the substantia nigra, and a portion was treated with protease K as described previously (Double *et al.* 2000). DAM and Cys-DAM were prepared by oxidation of DA in the absence and presence of L-cysteine as reported (Ben-Schachar *et al.* 1991). Melanin was suspended in 10 % dimethyl sulfoxide (DMSO) to be 1 mg/ml, sonicated for 30 s and shaken gently for 3 days for the rehydration at the room temperature under protection from light.

Measurement of apoptosis by morphological observation, FACS, and with LIVE/DEATH assay kit

SH-SY5Y cells were cultured in a 24-well poly-L-lysine-coated tissue culture plate and treated with melanin for 16 h at 37°C. After stained with 50 μM Hoechst 33342 or propidium iodide (PI), the cells were observed with a fluorescence microscope, Olympus Bx60 (Tokyo, Japan) equipped with an epi-illuminator. In other experiments, the cells

were cultured in 6-well culture flasks with melanin (10 μg/ml of Cosmedium-001) for 16 h. After gathered and washed with phosphate-buffered saline (PBS), the cells were stained with Hoechst 33342 (5 μg/ml) at 37°C for 20 min, washed twice with PBS, applied on a glass slide and observed with the fluorescence microscope.

Apoptosis was quantitatively assessed by fluorescence-augmented flow cytometry (FACS) with a FACScaliber 4A and CellQuest software (Benton Dickinson, San Jose, CA, USA). The cells cultured in a 6-well culture flask were treated with or without melanin (10-25 μg/ml in the final concentration) for 16 h. To determine apoptosis, the cells were stained with 75 μM PI solution in PBS containing 1% Triton X-100 at room temperature for 5 min in the dark, washed, suspended in PBS, then subjected to FACS analysis (Yi *et al.* 2006). The fluorescence intensity at 560-640 nm (FL-2 channel) was measured with excitation at 488 nm. Cells with a lower DNA content showing PI staining less than G_1 peak were defined to be apoptotic (subG_1 peak).

The cell viability was also measured using LIVE/DEAD viability/cytotoxicity kit according to the manufacture's instruction. The cells cultured in 6-well flasks, treated with melanin (5-25 μg/ml) for 16 h at 37°C, gathered by scraping, washed with PBS, and

suspended with Hanks' balanced salt solution. The cells were stained with 4 μM calcein AM to determine live cell number. Fluorescence intensity of calcein produced by esterase in live cells was measured at 485 nm with excitation at 530 nm in a Corona MTP-600F microplate fluorometer (Corona Electrics, Hitachinaka, Japan). The number of live cells were calculated by comparison of the fluorescence intensity of samples with that of control and expressed as % of the control. The protein content was measured according by Lowry method using DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

Measurement of mitochondrial membrane potential, ΔΨm, by FACS

ΔΨm in isolated mitochondria was quantified by FACS using $DiOC_6(3)$. Mitochondria were prepared from the wild and Bcl-2 cells, suspended in DMEM and incubated with 10-25 μg/ml NM or DAM at 37°C for 3 h. After stained with 25 nM $DiOC_6(3)$ for 15 min, the mitochondria were washed, suspended in PBS, and subjected to FACS. The laser emission at 560-640 nm (FL-2) with excitation at 488 nm was used for the detection of ΔΨm.

Western blot analysis for released cytochrome c

To detect cytochrome c released from mitochondria, the wild cells were incubated with melanin (10 μg/ml) for 1 to 6 h, gathered, washed with PBS, and treated with the extraction buffer [50 mM PIPES-KOH buffer, pH 7.4, containing 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol (DTT) and a complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany)]. The supernatant of centrifugation at 11 000 g for 20 min was mixed with an equi-volume of the RIPA buffer (10 mM Tris-HCl buffer, pH 7.5, containing 1% NM-40, 0.1% sodium dodecylcholate, 0.1% SDS, 150 mM NaCl and 1 mM EDTA). The sample (5 μg protein) was subjected to SDS-PAGE with a 10-20% polyacrylamide gel (Bio-Rad, Hercules, CA, USA), and electroblotted onto PVDF membranes (Du Pont, Boston, MA, USA). After blocking with nonfat milk, cytochrome c was visualized by use of antibodies against cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were treated with alkaline phosphatase-conjugated goat anti-mouse antibody (Promega, Madison, WI, USA) and visualized with an enhanced chemiluminescence detection kit (New England Biolabs, Beverly, MA, USA).

Assay for activities of caspase 3

The wild and Bcl-2 cells were cultured in 6-well tissue culture flasks in the presence of melanin (10-25 μg/ml) for 16 h at 37°C. The cells were gathered, washed with PBS, then lyzed in the lysis buffer (20 mM HEPES-KOH, pH 7.0, containing 10 mM KCl, 15 mM $MgCl_2$, 1 mM EDTA 2Na, 1 mM EGTA, 1 mM DTT, 250 mM sucrose and the protease inhibitor cocktail). The enzymatic activity was measured in the reaction mixture (20 mM HEPES buffer, pH 7.5, containing 10% glycerol and 2 mM DTT) with a substrate, Ac-DEVD-MCA (10 μM in the final concentration). After incubation at 37°C for 1 h, the fluorescence at 460 nm was measured with excitation at 360 nm in an MTP-600F microplate fluorometer. The fluorescence intensity was compared with standard 7-amino-4-methyl-coumarin (AMC) solution.

Determination of SH residue, GSH and GSSG

SH contents in mitochondria and melanin were quantitatively measured using Measure-iT™ Thiol assay kit, according the manufacture's instruction. The

fluorescence at 520 nm was measured with excitation at 490 nm in the microplate fluorometer. To differentiate the free and pellet-bound SH residues, mitochondria were treated with melanin (2 h at 37°C), incubated with 10 mM sodium phosphate buffer, pH 7.4, in an ice bath for 30 min, centrifuged at 12 000 g for 15 min, and the sediment was washed twice with the phosphate buffer by centrifugation. SH contents in the combined supernatant fractions and the pellets were quantified using the Thiol assay kit. GSH and GSSG in cells and mitochondria were also quantitatively measured using the enzymatic recycling method according to Rahman *et al.* (2006). The cells and mitochondria were centrifuged at 800 g for 5 min, or 12 000 g for 20 min, respectively, and the sediment was dissolved in the extraction medium (0.1 M potassium phosphate buffer, pH 7.5, containing 5 mM EDTA, 0.1% Triton X-100 and 0.6% sulfosalicylic acid). The supernatant of the centrifugation at 12 000 g for 20 min was subjected to the enzyme recycling assay. The cytosol and mitochondria fraction were prepared from the cells treated with 10 μg/ml NM, DAM or 100 μM dopamine for 2 h, according to Muyderman *et al.* (2004). HPLC with electrochemical detection was used to identify and quantify GSH in melanin-treated mitochondria, using conditions reported previously (Naoi *et al.*

1996). GSH was identified and quantified by comparison with GSH standard.

Detection of *S*-glutathionylated protein (PrS-SG) in mitochondria

S-Glutathionylate protein (PrS-SG) in mitochondria were detected by Western blot analysis after non-reducing SDS-PAGE, according to Brennan *et al.* (2004). Mitochondria were incubated with 10-25 μg/ml melanin for 2 h at 37°C, centrifuged at 12 000 x g for 10 min, and the pellets were washed twice with PBS. Mitochondria were suspended in the extraction buffer for PrS-SG (50 mM Tris-HCl buffer, pH 7.5, containing 5 mM EGTA, 2 mM EDTA, 100 mM NaF, 0.05% digitonin and 100 mM maleimide), allowed to stand in ice bath for 10 min, then centrifuged at 12 000 x g for 10 min. The extracts was reconstituted in SDS sample buffer containing 100 mM maleimide instead of reducing agents to block unreacted thiol group and subjected to SDS-PAGE. PrS-SG was visualized with anti-GSH antibody (Virogen, Watertown, MA, USA). To examine the reversibility of glutathionylation, parts of the samples were treated with the RIPA buffer containing 5% β-mercaptoethanol and subjected to SDS-PAGE under reducing conditions using SDS sample buffer. To identify

S-glutathionylated protein in mitochondrial complex I and III, the polyclonal antibodies against complex I and III were used (Tanaka *et al.* 1988).

Statistics

Experiments were repeated at least 3 times, and triplicate or quadruplicate measurements were carried out. The data were expressed as mean ± SD and the difference was evaluated by analysis of variance (ANOVA) followed by Scheffe's F-test. A p value less than 0.05 was estimated to be statistically significant.

Results

Apoptosis induced by NM

After incubation of SH-SY5Y cells with NM, apoptosis was detected by morphological observation. NM and dopamine induced cell death in the cells, and Hoechst 33342 staining showed apoptotic features with condensed nuclei among dead cells detected with PI (Fig. 1 A, Fig. 2 B). DAM was much less cytotoxic than NM and only few cells were positively stained with Hoechst 33342 and PI. The cytotoxicity of NM was