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Yan, Y., Nitta, A., Mizoguchi, H., Yamada, K. and Nabeshima, T.	Relapse of methamphetamine-seeking behavior in C57BL/6J mice demonstrated by a reinstatement procedure involving intravenous self-administration.	Behav. Brain Res.	168(1)	137-143	2006
Yan, Y., Nitta, A., Mizuno, T., Nakajima, A., Yamada, K. and Nabeshima, T.	Discriminative-stimulus effects of methamphetamine and morphine in rats are attenuated by cAMP-related compounds.	Behav. Brain Res.	173(1)	39-46	2006
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Niwa, M., Nitta, A., Shen, L., Noda, Y. and Nabeshima, T.	Involvement of glial cell-line derived neurotrophic factor inhibitory effects of a hydrophobic dipeptide Leu-Ile on morphine-induced sensitization and rewarding effects.	Behav. Brain Res.	In press		
Niwa, M., Nitta, A., Yamada, Y., Nakajima, A., Saito, K., Seishima, M., Shen, L., Noda, Y., Furukawa, S. and Nabeshima, T.	An inducer for glial cell line-derived neurotrophic factor and tumor necrosis factor- α protects against methamphetamine-induced rewarding effects and sensitization.	Biol. Psychiatry	In press		

T.					
Niwa, M., Nitta, A., Yamada, Y., Nakajima, A., Saito, K., Seishima, M., Shen, L., Noda, Y. and Nabeshima, T.	Tumor necrosis factor- α and its inducer inhibit morphine-induced rewarding effects and sensitization.	Biol. Psychiatry	In press		
Yan, Y., Yamada, K., Mizoguchi, H., Noda, Y., Nagai, T., Nitta, A. and Nabeshima, T.	Reinforcing effects of morphine are reduced in tissue plasminogen activator (tPA)-knockout mice.	Neuroscience	In press		
Yan, Y., Yamada, K., Niwa, M., Nagai, T., Nitta, A. and Nabeshima, T.	Enduring vulnerability to reinstatement of methamphetamine-seeking behavior in glial cell line-derived neurotrophic factor mutant mice.	FASEB J.	In press		

精神・神経変性疾患治療薬と 精神疾患関連遺伝子の発見

医学系研究科
細胞情報医学専攻
臨床薬物情報講座
医療薬学分野

准教授 新田 淳美 (にった あつみ)



一言アビール

神経栄養因子の研究をライフワークに選び、創薬に結びつく研究を行うことを研究者人生の目標としてきました。名古屋大学医学部附属病院教員への着任を機に神経変性・精神疾患治療薬の開発につながる研究を目指すようになりました。神経栄養因子の産生を誘導する化合物が、薬物依存・脊髄損傷・アルツハイマー病・パーキンソン病などに有効であることを示唆する結果を基礎研究で得ています。私達の研究グループでは、shati という新しい遺伝子(名古屋大学にて発見したことから、名古屋城の金の鯱にちなんで命名しました)と脳での機能について全く研究されていなかった piccolo という2つの遺伝子が精神疾患に関連していることを報告しました。これらの遺伝子の機能を調べて、神経・精神系の疾患の原因解明や治療法に結びつくことを目指しています。まずは、これら遺伝子が精神疾患の早期発見のマーカーとならないか調べ、産学連携研究を行いたく思っています。

研究のキーワード 薬物依存、行動薬理、神経再生、神経栄養因子、培養神経細胞、アルツハイマー病、パーキンソン病、酵素免疫測定法、遺伝子導入、抗体アレイ

保有技術・機器 (医療薬学講座 山田清文教授の研究室として)

- 【保有技術】・行動薬理学実験 (薬物嗜好性・学習記憶・情動性等) ・培養神経細胞への遺伝子導入
・神経栄養因子の高感度酵素免疫測定法 ・in vivo マイクロダイアリス ・cDNA サブトラクション
- 【主な機器】神経細胞へのエレクトロポレーション装置、蛍光透過型顕微鏡、蛍光顕微鏡、行動実験装置、吸光および蛍光プレートリーダー、リアルタイム RT-PCR、in vivo マイクロダイアリス装置、小型冷却超遠心機

主な特許・論文・著書

- 【主な特許】(海外特許への移行状況については本学知財部にお問い合わせください)
- ・特開 2002-300247 神経栄養因子誘導剤 ・特開 2003-279070 抗薬物依存形成剤 ・特願 2007-504648 Akt 刺激薬
 - ・特願 2007-505883 薬物依存関連遺伝子 ・特願 2006-287639 脳内酸化抑制剤およびその使用
- 【主な論文】
- ・ Identification of piccolo as a regulator of behavioral plasticity and dopamine transporter internalization. Mol. Psychiatry, In press
 - ・ A novel molecule 'shati' is involved in methamphetamine-induced hyperlocomotion, sensitization, and conditioned place preference. J. Neurosci., 27, 7604-7615 (2007)
 - ・ An inducer for glial cell line-derived neurotrophic factor and tumor necrosis factor- α protects against methamphetamine-induced rewarding effects and sensitization. Biol. Psychiatry, 61, 890-901 (2007)
 - ・ Tumor necrosis factor- α and its inducer inhibit morphine-induced rewarding effects and sensitization. Biol. Psychiatry, 62, 658-668 (2007)
 - ・ An analogue of dipeptide-like structure of FK506 increases GDNF expression through CREB activated by Hsp90/Akt signaling pathway. J. Neurosci., 26, 3335-3344 (2006)
 - ・ Hydrophobic dipeptide, Leu-Ile, protects against neuronal death by inducing brain-derived neurotrophic factor and glial cell line-derived neurotrophic synthesis. J. Neurosci. Res., 78, 250-258 (2004)

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- 平成 7年 名古屋大学大学院医学研究科生理系 医療薬学専攻修了
- 平成 7年 岐阜薬科大学薬学部分子生物学教室 助手
- 平成 13年 名古屋大学医学部附属病院薬剤部・大学院医学系研究科・医療薬学・助教授 (19年4月より呼称変更にて准教授)

研究01 精神疾患関連遺伝子

概要

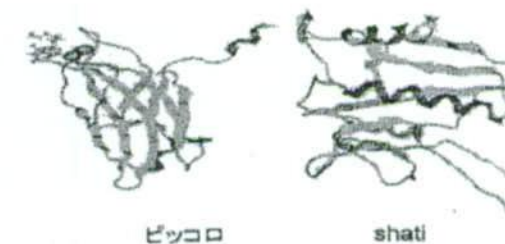
覚せい剤精神病モデルマウスの脳で過剰に発現している遺伝子を検索したところ、新規遺伝子 shati (名古屋大学発という意味を込めて、命名しました) と脳・神経系での研究がほとんどなされていない piccolo という遺伝子を見つけました。これらの遺伝子が精神疾患と関係していることが強く予想されるため、神経・精神疾患との関連について研究を進めている最中です。

この研究の新規性・独創性

shati と piccolo は、情動や運動機能、さらに人間の三大欲にも関係が深いドーパミンという神経伝達物質の遊離を調節することを見出しています。ドーパミンは統合失調症などの精神疾患やパーキンソン病のような神経変性疾患の key 物質でもあることから、本研究を進めれば、多くの精神・神経疾患の治療薬になる可能性があります。私たちは両タンパクの研究に必要な多くのツールをすでに作成・保有しています。

産学連携を目指した応用研究

覚せい剤精神病モデルマウスから見出した遺伝子であることから、精神疾患の早期発見マーカーとしての可能性を考えています。将来的には、脳疾患の遺伝子治療薬への応用を目指したいです。



研究02 多くの種類の神経疾患に対して効果のあるジペプチド

概要

私たち神経化学の研究者ですら神経幹細胞の概念が浸透するまでは、神経細胞は一旦形成されると2度と再生されることはなく、人間の寿命とともに神経細胞数は減少し、神経機能は衰えるのみと考えていました。神経栄養因子とよばれるタンパクは、神経細胞の死滅を防ぐ因子として大変注目されています。しかしながら、それらタンパクの多くは、大きな分子のため、血液中で分解されやすく、血液・脳関門の通過が難しいため、脳疾患の治療薬として用いることは不可能です。私たちは、神経栄養因子の産生を促進する低分子化合物 Leu-Ile が、神経変性疾患の治療薬として用いる可能性を示す研究成果を得ています。

この研究の新規性・独創性

Leu-Ile は、パーキンソン病、アルツハイマー病、薬物依存、脊髄ダメージを持っています。おもしろいことに、疾病の種類、すなわち生体側の状況によって異なる作用メカニズムが発揮されているようデータを持っています。おもしろいことに、疾病の種類、すなわち生体側の状況によって異なる作用メカニズムが発揮されているようです。

産学連携を目指した応用研究

Leu-Ile は化合物としてはシンプルのため、用途特許の出願が難しいようです。多くの作用を持ったジペプチドを世の中に出すお手伝いをしていただけませんか？



これまでの研究テーマ

- ・神経栄養因子の高感度酵素免疫測定法の確立
- ・神経栄養因子産生誘導物質のスクリーニング
- ・脊髄損傷治療法の確立への基礎研究
- ・薬物依存形成メカニズムの解明および治療薬の開発研究
- ・アルツハイマーおよびパーキンソン病治療薬の開発に向けての基礎研究
- ・ニコチンによる抗うつ作用
- ・アルツハイマー病モデルの開発および神経機能障害メカニズムの解明
- ・薬物依存および精神異常に関与する新規遺伝子クローニング
- ・ドーパミントランスポーター過剰発現細胞のクローニング
- ・うつ病治療薬の開発

The State of Drug Dependence

Associate Professor Atsushi Nitta / Neuropsychopharmacology and Hospital Pharmacy, Graduate School of Medicine

Abstract 1. A Novel Therapeutic Agent against Drug Dependence

Abstract

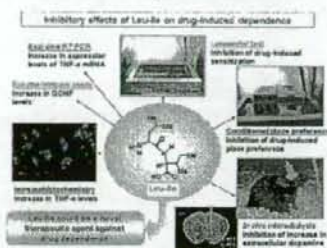
Leu-Ile, which induces neurotrophic factors, could be a novel therapeutic agent against not only neurodegenerative diseases such as Parkinson's disease and damage of the spinal cord, but also drug dependence by inhibiting drug-induced place preference and behavioral sensitization. Moreover, we have found that Leu-Ile inhibits neurodegenerative diseases and drug dependence by activating Akt, since Leu-Ile activates Akt signaling.

Novelty and Originality

There are few efficacious medications for drug dependence. Since we have found that Leu-Ile inhibits methamphetamine and morphine-induced dependence, we will investigate the effects of Leu-Ile on nicotine, alcohol, and other drugs of abuse-induced dependence in subsequent studies.

Applied Research Collaboration

Leu-Ile, a hydrophobic dipeptide, is safe, since mice show no toxicity for receiving 1g/mouse of Leu-Ile. Leu-Ile is contained in milk, salmon, red wine, etc. Leu-Ile would also be useful as a functional food as well as a medical drug, if Leu-Ile inhibits nicotine and alcohol-induced dependence.



Abstract 2. Involvement of Novel Molecules in Drug Dependence

Abstract

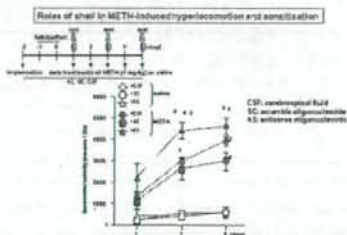
We have identified novel molecules from the nucleus accumbens of mice treated with methamphetamine. We have named one of the novel molecules "shati". The other has been identified as a diabetes-related protein, although there is no report of the physiological role it plays in the brain.

Novelty and Originality

We have found important molecules related to drug dependence. These molecules reduce dopamine release, which blocks drug dependence. We will investigate the relationship between these molecules and neuropsychological disorders.

Applied Research Collaboration

We are considering the application of diagnostic kits for several psychiatric diseases, based on the concentrations of the new proteins in blood.



Repeated METH administration leads to a progressive augmentation of many behavioral effects of the drug (behavioral sensitization). Sensitization is of interest as a model for drug-induced neuroplasticity in neuronal circuits important for addiction. It is well established that the induction of sensitization involves complex neuronal circuitry. Repeated METH treatment for 5 days produced locomotor sensitization. The AS treatment potentiated the development of locomotor sensitization to METH compared with -SC or CSF-treated mice.

Research Performance

Keywords

Drug dependence, Leu-Ile, Akt, Methamphetamine, Morphine, Nicotine, Alcohol, Dopamine, Mice

Publications

- A novel molecule 'shati' is involved in methamphetamine-induced hyperlocomotion, sensitization, conditioned place preference. *Journal of Neuroscience*, in press.
- Tumor Necrosis Factor- α and Its Inducer Inhibit Morphine-Induced Rewarding Effects and Sensitization. *Biological Psychiatry*, 2007 Jan 9; [Epub ahead of print]
- An inducer for glial cell line-derived neurotrophic factor and tumor necrosis factor- α protects against methamphetamine-induced rewarding effects and sensitization. *Biological Psychiatry*, 61:890-901, 2007
- An analog of a dipeptide-like structure of FK506 increases glial cell line-derived neurotrophic factor expression through cAMP response element-binding protein activated by heat shock protein 90/Akt signaling pathway. *Journal of Neuroscience*, 26:3335-3344, 2006

International Patents

- PCT/JP2006/301326 A reagent for Akt activation
- PCT/JP2006/303376 Novel genes and proteins-related to psychiatric diseases.

International Research Career

- 2001-2002 Douglas Hospital Research Center, Department of Psychiatry, McGill University, Verdun-Montreal, Canada "Development new drugs for Alzheimer's disease"

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

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Abstract

Parkinson's disease (PD) is characterized by selective depletion of nigral dopamine (DA) neurons containing neuromelanin (NM), suggesting the involvement of NM in the pathogenesis. This study reports induction of apoptosis by NM in SH-SY5Y cells, whereas protease-K-treated NM, synthesized DA- 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 over-expression 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 DA neurons in PD 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 PD.

Keywords: apoptosis, mitochondria, neuromelanin, Parkinson's disease, Redox status, S-glutathionylation.
J. Neurochem. (2008) **105**, 2489–2500.

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

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Abbreviations used: $\Delta\Psi_m$, mitochondrial membrane potential; Cys-DAM, cysteinyl dopamine melanin; DAM, dopamine melanin; DFX, deferoxamine mesylate; EGCG, (–)-epigallocatechin gallate; FACS, fluorescence-augmented flow cytometry; GRX, glutaredoxin; mPT, mitochondrial permeability transition; NM, neuromelanin; NO, nitric oxide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PD, Parkinson's disease; P-K NM, protease-K-treated neuromelanin; PrS-SG, S-glutathionylated protein; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SH, sulfhydryl; SOD, superoxide dismutase; TRX, thioredoxin.

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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 autooxidation of dopamine (DA), noradrenaline, 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 : 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-DA and -DOPA derived by conjugation of DA 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 DA 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 C₁₄–C₁₈ fatty acids and dolichol (Zecca *et al.* 2000; Fedorow *et al.* 2005). NM also contains 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 DA neurons through the production of reactive oxygen and nitrogen species (ROS and RNS) (Ben-Shachar *et al.* 1991) and of cytokines and nitric oxide (NO) 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 DA 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 DA quinone (Youdim *et al.* 1994).

Human NM and synthetic melanin from DA (dopamine melanin; DAM) or L-DOPA, induced cell death in rat pheochromocytoma 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 (Offen *et al.* 1997; Li *et al.* 2005), 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 in 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 might be because of requirement for reducing conditions to activate caspase 3, an apoptosis executor, by caspase 8 (Hentze *et al.* 2000), and to suppress nuclear factor- κ B 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 sulfhydryl (SH) residue of GSH (*S*-glutathionylated protein; PrS-SG) (Maher 2006). This post-translational modification of protein, referred as *S*-glutathionylation, regulates the essential cellular functions, such as energy synthesis, signal transduction pathway, and transcriptional activation (Schäfer and Büttner 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 DA neurons.

In this study, 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 protease K (P-K NM), and eumelanin and pheomelanin were synthesized by oxidation of DA in the absence (DAM) or presence of L-cysteine (cysteinyldopamine melanin; Cys-DAM). Wild and transfection-enforced Bcl-2 over-expressed 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 DA neurons containing NM.

Materials and methods

Materials

3,3'-Dihydroxycarbocyanide iodide [DiOC₆(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), and (-)-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), was obtained from Peptide Institute (Osaka, Japan). Catalase from bovine liver, Dulbecco's minimum essential medium 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% CO₂. Bcl-2 was over-expressed in SH-SY5Y cells (Bcl-2 cells) by transfection as reported previously (Akao *et al.* 2002).

Preparation of neuromelanin and dopamine melanin

Brains from neurologically normal adult individuals were provided from the Austro-German Brain Bank in Würzburg, Germany. The Ethics Committee of the University Clinics of Würzburg (Würzburg, Germany) approved the use of postmortem human brain tissue. NM was isolated from the substantia nigra, and a portion was treated with P-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-Shachar *et al.* 1991). Melanin was suspended in 10% dimethyl sulfoxide to be 1 mg/mL, sonicated for 30 s, and shaken gently for 3 days for the rehydration at 20°C under protection from light.

Measurement of apoptosis by morphological observation, FACS, and with LIVE/DEAD 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 μ M 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 six-well culture flasks with melanin (10 μ g/mL of Cosmedium-001) for 16 h. After gathered and washed with phosphate-buffered saline (PBS), the cells were stained with Hoechst 33342 (5 μ g/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 six-well culture flask were treated with or without melanin (10–25 μ g/mL in the final concentration) for 16 h. To determine apoptosis, the cells were stained with 75 μ M PI solution in PBS containing 1% Triton X-100 at 20°C for 5 min in the dark, washed, suspended in PBS, and then subjected to FACS analysis (Yi *et al.* 2006). The fluorescence intensity at 560–640 nm (fluorescence detector #2, FL-2 channel) was measured with excitation at 488 nm. Cells with a lower DNA content showing PI staining less than G₁ peak were defined to be apoptotic (subG₁ peak).

The cell viability was also measured using LIVE/DEAD viability/cytotoxicity kit according to the manufacturer's instruction. The

cells cultured in six-well flasks, treated with melanin (5–25 μ g/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 μ M 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 Electronics, Hitachinaka, Japan). The number of live cells were calculated by comparison of the fluorescence intensity of samples with that of control and expressed as percentage 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 by fluorescence-augmented flow cytometry

Mitochondrial membrane potential ($\Delta\Psi$ m) in isolated mitochondria was quantified by FACS using DiOC₆(3). Mitochondria were prepared from the wild and Bcl-2 cells, suspended in Dulbecco's minimum essential medium, and incubated with 10–25 μ g/mL NM at 37°C for 3 h. After stained with 25 nM DiOC₆(3) for 15 min, the mitochondria were washed, suspended in PBS, and subjected to FACS. The laser emission at 560–640 nm (fluorescence detector, FL-2 channel) with excitation at 488 nm was used for the detection of $\Delta\Psi$ m.

Western blot analysis for released cytochrome c

To detect cytochrome c released from mitochondria, the wild cells were incubated with melanin (10 μ g/mL) for 1–6 h, gathered, washed with PBS, and treated with the extraction buffer [50 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES)-KOH buffer, pH 7.4, containing 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 2 mM MgCl₂, 5 mM EGTA, and 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 sample buffer [10 mM Tris-HCl buffer, pH 7.5, containing 1% Nonidet P-40 (Wako, Osaka, Japan), 0.1% sodium dodecyl cholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, and 1 mM EDTA]. The sample (5 μ g protein) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with a 10–20% polyacrylamide gel (Bio-Rad), and electroblotted onto polyvinylidene difluoride membranes (Du Pont, Boston, MA, USA). After blocking with non-fat 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 six-well tissue culture flasks in the presence of melanin (10–25 μ g/mL) for 16 h at 37°C. The cells were gathered, washed with PBS, then lysed in the lysis buffer (20 mM HEPES-KOH, pH 7.0, containing 10 mM KCl, 15 mM MgCl₂, 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, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid α -(4-methyl-coumaryl-7-amide) (10 μ M 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 solution.

Determination of SH residue, GSH, and GSSG

Sulfhydryl contents in mitochondria and melanin were quantitatively measured using Measure-IT™ Thiol assay kit, according to the manufacturer'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 at 12 000 g for 20 min. 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 μ g/mL NM, DAM, or 100 μ M DA for 2 h, according to Musderman 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 in mitochondria

S-Glutathionylated protein 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 μ g/mL melanin for 2 h at 37°C, centrifuged at 12 000 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 g for 10 min. The extracts were 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 SDS sample buffer containing 5% β -mercaptoethanol and subjected to SDS-PAGE. To identify PrS-SG 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 three times, and triplicate or quadruplicate measurements were carried out. The data were

expressed as mean \pm SD and the difference was evaluated by ANOVA followed by Scheffe's *F*-test. A value of *p* < 0.05 was estimated to be statistically significant.

Results

Apoptosis induced by neuromelanin

After incubation of SH-SY5Y cells with NM, apoptosis was detected by morphological observation. NM and DA induced cell death in the cells, and Hoechst 33342 staining showed apoptotic features with condensed nuclei among dead cells detected with PI (Figs. 1a and 2b). DAM was much less cytotoxic than NM and only few cells were positively stained with Hoechst 33342 and PI. The cytotoxicity of NM was quantitatively analyzed by FACS and apoptotic cells were detected as the subG₁ peak (Fig. 1b). NM treatment increased the number of apoptotic cells significantly to 37.9% of the total cells from 2.23% in control, whereas DAM induced apoptosis in 11.6% cells. Figure 1c shows the effects of NM and DAM concentrations on the viability of the wild and Bcl-2 cells as measured quantitatively using FACS. NM at concentrations higher than 10 μ g/mL induced apoptosis in the wild cells in a dose-dependent way. Transfection-enforced Bcl-2 overexpression did not prevent apoptosis caused by NM and DAM.

Effects of melanin species and protein component of NM on the cytotoxicity

Neuromelanin contains protein component in addition to mixed melanin of black eumelanin and brown pheomelanin. SH residues were detected in NM as measured with the fluorometric Thiol assay kit, which can detect GSH, cysteine, and related SH compounds in free and protein-bound form. SH content in NM was determined to be 2.42 ± 0.80 nmol/mg melanin, whereas SH was not detected in DAM (Table 1). P-K NM reduced the SH contents significantly to 0.57 ± 0.16 nmol/mg melanin, 23% of the untreated NM. In Cys-DAM synthesized from DA in the presence of L-cysteine, high SH content was determined, 3.77 ± 0.19 nmol/mg melanin.

The cytotoxicity of these melanin species was quantitatively measured by calcein staining for live cells (Fig. 2a). Among four melanin classes, only NM reduced the number of live cells, and the P-K treatment suppressed the NM cytotoxicity. DAM and Cys-DAM were much less cytotoxic than NM and the difference from control was not statistically significant. The cell death was confirmed by histopathological observation after staining with Hoechst 33342 (Fig. 2b). NM and DA induced apoptosis with the typical condensation and fragmentation of nucleus in most of the cells, while P-K NM, DAM, and Cys-DAM virtually did not induce cell death.

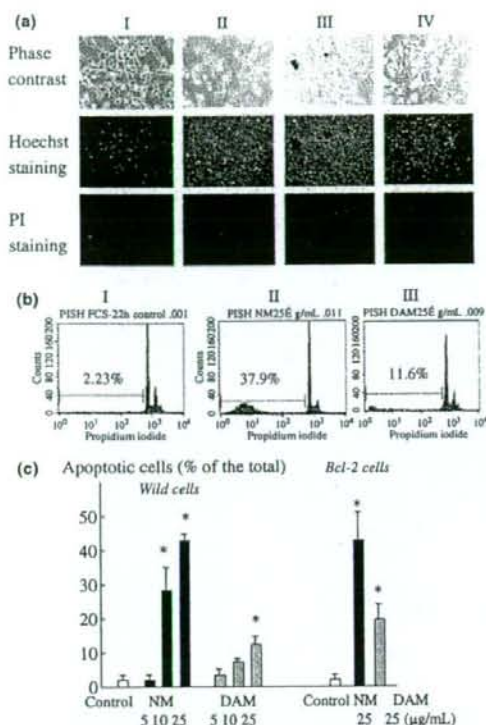


Fig. 1 Apoptosis induced by neuromelanin. (a) Morphological observation of NM cytotoxicity. SH-SY5Y cells were treated without (I, control) or with 10 µg/mL of NM (II), DAM (III), and 100 µM dopamine (IV) for 16 h. The cells were observed by phase contrast, or after staining with Hoechst 33342 for apoptotic cells and with PI for dead cells. (b) FACS analyses of apoptosis. The wild cells were incubated without (I, control) or 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.

Apoptosis pathway activated by neuromelanin

Mitochondria were prepared from the wild and Bcl-2 cells, treated with NM, and then subjected to FACS analyses after stained with DiOC₆(3). Figure 3a shows that NM reduced $\Delta\Psi_m$ of the wild cells in a dose-dependent way, which GSH did not prevent. In mitochondria prepared from Bcl-2 cells, the reduction of $\Delta\Psi_m$ by NM was also observed (Fig. 3b).

After treated with NM, cytochrome *c* was released from mitochondria into cytosol in wild SH-SY5Y cells in a time-dependent way (Fig. 3c). The cells were treated with NM,

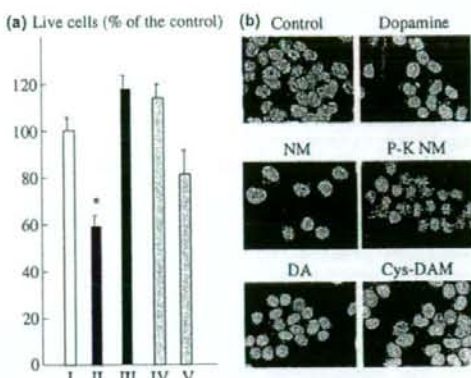


Fig. 2 Effects of protein component and melanin classes on cell viability. SH-SY5Y cells were cultured in a six-well tissue culture flask and treated without (I) or with 10 µg/mL NM (II), P-K NM (III), DAM (IV), or Cys-DAM (V) for 16 h at 37°C. (a) The number of live cells. The cells were gathered, washed 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).

Table 1 SH contents in NM, P-K-treated NM, DAM, and cysteinyl-DAM

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

SH contents in three NM and two P-K NM samples from different brains were measured fluorometrically by use of Measure-iT™ assay kit. The values are mean ± SD of quadruplicate measurements of each sample. NM, neuromelanin; SH, sulphydryl; P-K NM, protease-K-treated neuromelanin; DAM, dopamine melanin.

DAM, and DA for 16 h, and the activity of caspase 3 was measured fluorometrically. Figure 3d shows the significant increase in caspase 3 activity in the wild cells treated with NM and DA. On the other hand, DAM treatment did not affect the activity. In Bcl-2 cells, increase in caspase 3 activities was much less than in the wild cells, but NM still increased the activity markedly. Bcl-2 over-expression completely prevented the increase in caspase 3 activity by DA.

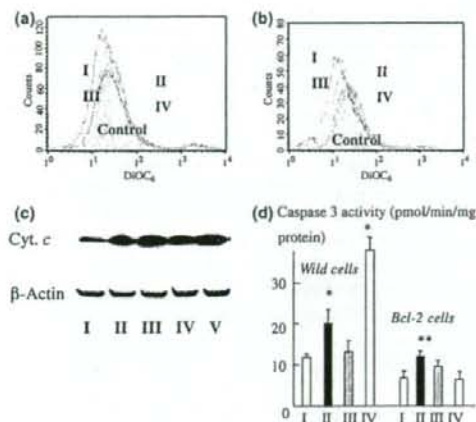


Fig. 3 Activation of apoptotic cascade by NM. (a and b) Reduction of $\Delta\Psi_m$ by NM in mitochondria isolated from the wild (a) and Bcl-2 cells (b). I and II, NM (25 and 10 $\mu\text{g}/\text{mL}$) was incubated with mitochondria without or with 1 mM $\text{DiOC}_6(3)$ (III and IV), respectively, at 37°C for 3 h. $\Delta\Psi_m$ was measured by FACS after staining with $\text{DiOC}_6(3)$. (c) Release of cytochrome c into cytosol. After treated with NM (25 $\mu\text{g}/\text{mL}$) for 6 h, cytosol fraction was prepared from SH-SY5Y cells and subjected to western blot analysis for cytochrome c (Cyt. c). I, Control and II, III, IV, and V, cells were treated with NM for 1, 2, 4, and 6 h. β -Actin 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 and II, III, and IV, cells treated with 10 $\mu\text{g}/\text{mL}$ NM and DAM, and 100 μM dopamine, respectively. Caspase 3 activity was measured fluorometrically using acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid α -(4-methyl-coumaryl-7-amide) as a substrate. The column and bar represent the mean and SD of triplicate measurements of 4 experiments. *Difference from control, $p < 0.01$.

Effects of neuromelanin and dopamine melanin on SH state in mitochondria

Effects of NM and DAM on mitochondrial SH levels were examined. Mitochondria prepared from the wild and Bcl-2 cells were incubated with NM (25 $\mu\text{g}/\text{mL}$) and SH contents were measured for 3 h, and NM significantly increased SH levels in mitochondria from 30 min to 2 h and reached to a plateau (Fig. 4a). On the other hand, DAM (25 $\mu\text{g}/\text{mL}$) and DA (100 μM) significantly reduced SH contents in a time-dependent way (Fig. 4b). The effects of four melanin species on mitochondrial SH levels are shown in Fig. 4c. P-K NM did not increase SH, while DAM reduced SH after 2 h incubation. The increased SH residues were identified to be GSH by use of HPLC, as shown in Fig. 4d. NM significantly increased GSH levels in mitochondria, whereas P-K NM did not affect and DAM reduced GSH levels, as in the case of the total SH contents measured by the fluorescent assay.

To confirm the localization of NM-increased SH compounds in submitochondrial fractions, mitochondria were

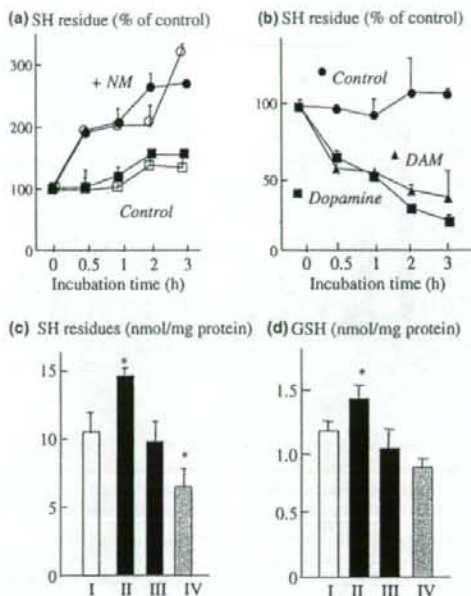


Fig. 4 Effects of melanin on SH levels in mitochondria. (a) Effects of NM on the mitochondrial SH contents. Mitochondria prepared from the wild (filled circle) and Bcl-2 cells (hollow circle) were incubated with NM (25 $\mu\text{g}/\text{mL}$) at 37°C , for 0.5, 1, 2, and 3 h. Mitochondria were treated 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 percentage 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 $\mu\text{g}/\text{mL}$) (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 $\mu\text{g}/\text{mL}$ 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 two experiments. *Difference from control, $p < 0.05$. (d) GSH was quantitatively measured by HPLC. Mitochondria were treated without (I, control) or with 25 $\mu\text{g}/\text{mL}$ 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 two experiments. *Difference from control, $p < 0.05$.

treated with these melanins, and differentiated into the soluble fraction and the precipitate. After NM-treatment, the SH contents increased significantly in both the fractions, whereas DAM and especially DA reduced SH contents (Fig. 5a). Cys-DAM markedly increased SH in the soluble fraction and pellets.

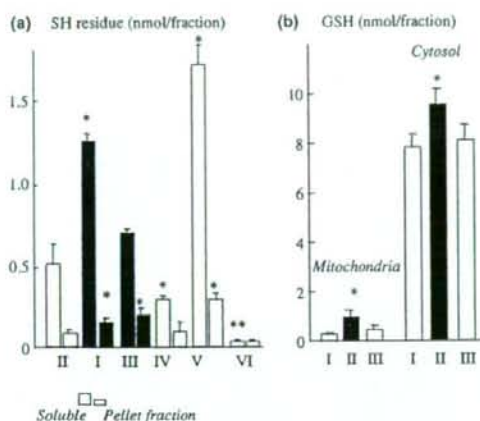


Fig. 5 Effects of NM and DAM on SH levels in mitochondria and subcellular fractions. (a) Effects of melanin species on SH levels in the soluble and precipitate fraction of mitochondria. Mitochondria prepared from SH-SY5Y cells were incubated without (I) or with 25 $\mu\text{g}/\text{mL}$ NM (II), P-K NM (III), DAM (IV), Cys-DAM (V), and 250 μM dopamine (VI) for 2 h at 37°C, then subjected to fractionation into the supernatant fraction and the sediment. SH levels were measured with Thiol Assay Kit and expressed as nmol/fraction. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *Difference from control, $p < 0.01$. (b) Effects of melanin on SH levels in cytosol and mitochondria fractions. The wild cells were treated with 25 $\mu\text{g}/\text{mL}$ of NM and DAM at 37°C for 2 h and subjected to subcellular fractionation. Mitochondrial and cytosol fraction were prepared for GSH analysis by GSH reductase-dependent recycle method. I, Control. II and III, cells treated with NM and DAM. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *Difference from control, $p < 0.01$ from control.

Neuromelanin- and DAM-treated cells were fractionated into mitochondria and cytosol fraction, and GSH contents were quantified by the enzyme recycling method (Fig. 5b). NM significantly increased GSH in mitochondrial and cytosol fraction, but DAM did not affect GSH levels.

Table 2 summarizes the total SH, GSH, and GSSG contents, and the GSSG/GSH ratio in the wild cells, the subcellular fractions and the isolated mitochondria after NM- and DAM-treatment. NM increased the total SH and GSH contents in the cells, the mitochondria fraction and the isolated mitochondria, significantly ($p < 0.05$), but not in the cytosol. GSSG levels were also increased in these samples after treated with NM. DAM reduced the total SH and GSH contents and increased GSSG levels in the mitochondrial fraction and the isolated mitochondria. NM increased significantly the GSSG/GSH ratio in the cell lysate, mitochondrial fraction and isolated mitochondria and DAM increased the ratio more markedly.

Effects of antioxidant on the cytotoxicity and SH reduction by NM

The involvement of ROS-RNS in NM-induced cell death and increase of mitochondrial SH levels was examined. After incubated with NM in the presence of antioxidants, the cell viability was quantitatively measured by calcein staining. Iron-chelating DFX, SOD, and NO-scavenging EGCG protect cells from cell death induced by NM, but catalase did not (Fig. 6a). At the same time, DFX, SOD, and EGCG prevented the NM-induced increase in mitochondrial SH contents, but catalase further increased SH levels (Fig. 6b).

The effects of NADPH-dependent recycling system on the NM-increased SH levels were examined in mitochondria. NADPH enhanced NM-induced increase in SH levels, but did not affect the levels in control and DAM-treated mitochondria.

Effects of NM on S-glutathionylated protein in mitochondria

To find the origin of GSH increased in mitochondria by NM, mitochondria prepared from the wild cells were treated with NM and other melanin (10 $\mu\text{g}/\text{mL}$), and the protein was subjected to western blot analysis for PrS-SG. Under non-reducing conditions, PrS-SG was detected in mitochondria without NM treatment (Fig. 7a). NM reduced PrS-SG especially with high molecular mass, while other melanin increased PrS-SG. Under reducing conditions, reduction of PrS-SG was confirmed again in NM-treated mitochondria, where PrS-SG proteins with molecular mass higher than 50 kDa were reduced significantly (Fig. 7b). Mitochondrial complex I proteins were visualized with anti-complex I antibody. Under non-reducing conditions, NM treatment disaggregated the macromolecular structure of complex I. Complex I proteins with 100–150 kDa disappeared, and complex I subunits with less than 75 kDa increased significantly. On the other hand, other melanin did not affect the high structure of complex I. Under reducing condition the amount and electrophoresis-pattern of complex I subunits did not change by NM-treatment, as shown by western blot analyses, indicating the reversibility of NM-induced dissociation of complex I subunits. Under non-reducing conditions, the reactivity against anti-complex III antibody was slightly reduced by NM treatment, but other melanin did not affect the amounts and pattern of complex III subunits. In mitochondria prepared from Bcl-2 cells, the same results were obtained.

Discussion

This study presents a novel role of NM in the pathogenesis of PD. NM induces apoptosis in SH-SY5Y cells through activation of death cascade, which depends on the protein component, SH content and melanin species. In PD NM contents in the substantia nigra reduced to

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 × 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
Treated with 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
Treated with 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
Treated with 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
Treated with 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 Musderman *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 at 12 000 g for 20 min, treated as above for GSH-GSSG assay. NM, neuromelanin; SH, sulphydryl; DAM, dopamine melanin.

1.2–1.5 mg/g wet weight from 2.3 to 3.5 mg/g wet weight (Zecca *et al.* 2001), as indicated by loss of dark brown color in this region. However, it remains unclear whether the protein, lipid, and inorganic components of NM change the nature in PD. Protein associated with NM granules from normal brains was subjected to the proteomic studies and about 70 kinds of protein were detected, but the accumulation of specified proteins was not confirmed (Tribl *et al.* 2005). In PD brain, α -synuclein is associated with NM (Fasano *et al.* 2003), and NM isolated from PD brains is composed mainly of highly cross-linked, protease resistant protein-like materials (Aime *et al.* 2000). Considering the increase of oxidative stress in parkinsonian brains, proteins in NM granules might be highly modified with ROS–RNS and tend to be more aggregated, which may be accelerated further by dysfunction of the ubiquitin-proteasome system (McNaught *et al.* 2001). However, the cytotoxicity of NM-associated protein itself has never been reported in NM prepared from either normal or parkinsonian brains. In A9 neurons of PD brains, the loss of cholesterol and the aggregation of α -synuclein to lipid in NM were reported by histopathological observation (Halliday *et al.* 2005). However, the lipid components may not be involved in the toxicity of NM observed in this study, as lipid is washed out during the purification procedure (Double *et al.* 2000).

The role of protein component in the NM toxicity was clearly demonstrated in this study. P-K treatment totally reduced NM cytotoxicity, and synthesized DAM and Cys-DAM did not induce the cell death in SH-SY5Y cells under used conditions. In NM, SH residues were detected as exposed on the surface, which P-K-treatment reduced to one fourth, suggesting that a major part of SH groups is derived from the protein and the rest from the pheomelanin component. This result may be comparable with the previous result that P-K-treatment reduced amino acid contents from 165 to 57 µg/mg melanin (Double *et al.* 2000). According to the reported amino acid composition, the cysteine content of NM is 10.6 ± 3.7 nmol/mg melanin (Double *et al.* 2000). SH level in NM is quantified to be 2.42 ± 0.80 nmol/mg melanin, suggesting that most of cysteine is sequenced in protein as intra- or inter-disulfide bond, or occurs as the mixed disulfide bond between protein SH and GSH, cysteine, or related SH derivatives. In pheomelanin produced from 5-S-cysteinyl-DA a conjugate of cysteine with *o*-quinone, free SH residues were detected, even though pheomelanin has been considered to polymerize into a benzothiazine structure.

Neuromelanin and DAM affected SH state in mitochondria in quite opposite ways. NM and Cys-DAM increased SH contents in mitochondria, whereas DAM and DA reduced them markedly. These results might be comparable to the

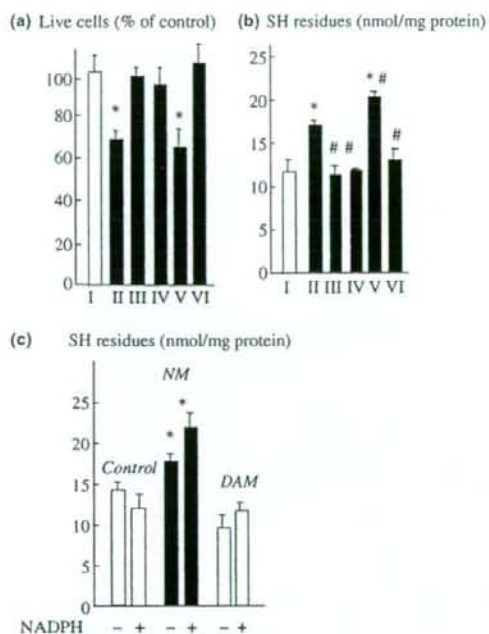


Fig. 6 Effects of antioxidants and NADPH on the NM-induced apoptosis and SH increase. (a) Effects of antioxidants on cell death induced by NM. The wild cells were treated without (I, control) or with NM alone (II) or NM in the presence of DFX (III, 1 μ M), SOD (IV, 100 U/mL), catalase (V, 300 U/mL), and EGCG (VI, 10 μ M) for 16 h at 37°C. Live cells were quantified using calcein staining. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *Difference from control, $p < 0.05$ from control. #Difference from NM-treated cells, $p < 0.05$. (b) Effects of antioxidants on SH levels in mitochondria. Mitochondria were treated without or with NM in the absence (I, control) or presence of DFX (II, 1 μ M), SOD (III, 100 U/mL), catalase (V, 300 U/mL), and EGCG (VI, 10 μ M) for 2 h at 37°C. SH contents were measured fluorometrically using the Thiol Assay Kit. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *Difference from control, $p < 0.05$. #Difference from NM-treated cells, $p < 0.05$. (c) Effects of NADPH on SH contents in melanin-treated mitochondria. Mitochondria were treated without (Control) or with 25 μ g/mL NM or DAM at 37°C for 2 h, in the absence (-) or presence (+) of 1 mM NADPH. SH was quantified with the Thiol Assay Kit. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *Difference from control, $p < 0.01$. #Difference from NM-treated cells, $p < 0.05$.

previous results that DAM increased ROS-RNS and induced cell death in neuronal SK-N-SH cells, whereas NM protected the cells from hydroxyl radicals produced by the Fenton reaction (Li *et al.* 2005). Melanosomes containing eumelanin and pheomelanin have oxidation potentials of -0.2 and

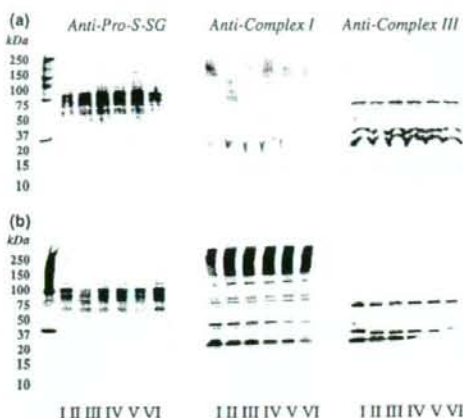


Fig. 7 Effects of NM on S-glutathionylated protein in mitochondria. Mitochondria were prepared from the wild cells and treated without (I, control), or with 10 μ g/mL NM (II), P-K NM (III), DAM (IV), Cys-DAM (V), or 100 μ M dopamine (VI) at 37°C for 2 h. The samples were washed with PBS, and subjected to SDS-PAGE under non-reducing (a) and reducing conditions (b). S-Glutathionylated protein (PrS-SG) was visualized by use of polyclonal anti-GSH antibody. Complex I and III were detected with the antibody against complex I and III, respectively. The left line of each gel represents the protein markers with molecular mass with 250, 150, 100, 75, 50, 37, 20, 15, and 10 kDa from the top.

+0.5 V, respectively (Samokhvalov *et al.* 2005). Pheomelanin in NM and Cys-DAM may reduce disulfide bonding and release GSH or cysteine from the mixed disulfide bonding and increases SH content in mitochondria, while eumelanin and DA oxidatively modify SH residues. These results are relevant with the fact that NM has the surface oxidation potential different from synthesized DAM (Bush *et al.* 2006).

The role of NM-associated protein was examined in concern to regulation of mitochondrial 'redox state,' which is related to many physiological and pathological phenomena of cells (Schafer and Buettner 2001; Maher 2006). Redox state depends on the reduction potential of redox pairs, GSH/GSSG, reduced/oxidized thioredoxin [Trx(SH)₂/TrxSS], and NADPH/NADP⁺. GSH/GSSG pool is the largest in cells and a major indicator of the redox state. Most glutathione is localized in the cytosol, but there is a GSH pool in mitochondria, which accounts for about 15% of the total (Meister 1995). GSH reduces thiol modifications of disulfide bonding and thioesters, and it is a substrate of protein S-glutathionylation. S-Glutathionylation is reversible and transitional in cytosol, where mM order of GSH and ascorbic acid are present, but in mitochondria with high oxidative environments (Table 2), S-thiolated proteins are more stabilized and detected in complex I, as

reported here and by Taylor *et al.* (2003). Glutathionylated NADP⁺-dependent isocitrate dehydrogenase was detected in brains from a PD model of MPTP-treated mice (Kil and Park 2005). Under oxidative conditions, actin, glyceraldehyde-3-phosphate dehydrogenase, protein kinase, heat-shock protein 27, protein-tyrosine phosphatase 1B, and protein kinase C α are the substrate of S-thiolation (Eaton *et al.* 2002). In physiological conditions or mild oxidative stress, thiols in protein (PrS-SG) or GSH are reversibly modified into active intermediates, such as thiolate, sulfenate, and sulfenic acid, by NO, superoxide, hydrogen peroxide, and peroxynitrite (Klatt and Lamas 2000). The activated protein SH groups reacts with GSH, or *vice versa* activated GSH reacts with reduced protein SH to generate GSH-protein mixed disulfide (PrS-SG). Prolonged or intense generation of ROS-RNS irreversibly oxidized thiols into protein cysteic acid by sulfonation, which was detected in the brains of patients with PD and Alzheimer's diseases (Choi *et al.* 2005). Glutathionylation is reversed by glutaredoxin (GRX), other TRX, and protein disulfide isomerase (EC 5.3.4.1), and yields free SH in protein (Pr-SH) and GSH from PrS-SG. This reaction is recycled by TRX reductase (EC 1.6.4.5) or GSH reductase (EC 1.6.4.2) using NADPH as a cofactor.

Neuromelanin significantly reduces PrS-SG detected in mitochondria, especially in complex I (Fig. 7). In addition, NM dissociates high structure of complex I into the subunits, and the dissociation is reversed by reducing agents, suggesting that S-glutathionylation stabilizes the high structure of complex I under physiological condition. It may be reasonable to consider that SH group of NM functions as that in GRX, reduces the disulfide bonding in mitochondrial protein, release free GSH, or cysteine and exposes free protein SH. Only NM, but not Cys-DAM, reduces mitochondrial PrS-SG, indicating that NM-associated protein, not pheomelanin, affects the mixed disulfide bonding in mitochondria. The SH amounts in NM used for these experiments were less than 1% of the total SH content present in mitochondria, 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 over-expression did not prevent cell death, even though Bcl-2 protects the cells against apoptosis induced by DA (Fig. 3d) 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(N,N'-dimethylamide) (diamide)

induced mitochondrial permeability transition (mPT) by modifying thiol of Cys57 in adenine nucleotide translocator (ANT) localized on the matrix site at the cyclosporine-binding site (Costantini *et al.* 2000). Modification of Cys57 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 study 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 mitochondria may reproduce those in the substantia nigra of aged and parkinsonian brains, where the redox state tends to more oxidizing condition with reduction of GSH levels. In normal condition the compensative antioxidant capacity might prevent the activation of death process by NM, even though it accumulates in the substantia nigra at the concentrations of 2–4 $\mu\text{g}/\text{mg}$ wet weight, almost the same levels as used in this study, 10–25 $\mu\text{g}/\text{mL}$ 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 DA 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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Formation of Dopamine Adducts Derived from Brain Polyunsaturated Fatty Acids

MECHANISM FOR PARKINSON DISEASE^{†‡}

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Oxidative stress appears to be directly involved in the pathogenesis of the neurodegeneration of dopaminergic systems in Parkinson disease. In this study, we formed four dopamine modification adducts derived from docosahexaenoic acid (C22:6/ ω -3) and arachidonic acid (C18:4/ ω -6), which are known as the major polyunsaturated fatty acids in the brain. Upon incubation of dopamine with fatty acid hydroperoxides and an *in vivo* experiment using rat brain tissue, all four dopamine adducts were detected. Furthermore, hexanoyl dopamine (HED), an arachidonic acid-derived adduct, caused severe cytotoxicity in human dopaminergic neuroblastoma SH-SY5Y cells, whereas the other adducts were only slightly affected. The HED-induced cell death was found to include apoptosis, which also seems to be mediated by reactive oxygen species generation and mitochondrial abnormality. Additionally, the experiments using monoamine transporter inhibitor and mouse embryonic fibroblast NIH-3T3 cells that lack the monoamine transporter indicate that the HED-induced cytotoxicity might specially occur in the neuronal cells. These data suggest that the formation of the docosahexaenoic acid- and arachidonic acid-derived dopamine adducts *in vitro* and *in vivo*, and HED, the arachidonic acid-derived dopamine modification adduct, which caused selective cytotoxicity of neuronal cells, may indicate a novel mechanism responsible for the pathogenesis in Parkinson disease.

Parkinson disease (PD)³ is a neurodegenerative disorder characterized by a dramatic loss of dopaminergic neurons in

the substantia nigra and the subsequent deficiency of dopamine in the brain areas (1). Until now, very little is known about why and how the PD neurodegenerative process begins and progresses; however, an increasing body of evidence suggests that oxidative stress, mitochondrial dysfunction, and impairment of the ubiquitin-proteasome system may be involved in the pathogenesis of PD (2–5). Recent studies indicate that there are high levels of basal oxidative stress in the substantia nigra pars compacta in the normal brain, and this is increased in PD (6).

Oxidative stress in the brain easily leads to the lipid peroxidation reaction because of a high concentration of polyunsaturated fatty acids, such as docosahexaenoic acid (DHA, C22:6/ ω -3) and arachidonic acid (AA, C18:4/ ω -6), which are present in the brain (7). The polyunsaturated fatty acids are located almost exclusively in the SN2 position of the phosphoglycerides found in the neural cell membranes. The beneficial physiological effects of DHA and AA have been frequently reported (8, 9); however, the fatty acids are highly unsaturated, thus making them particularly susceptible to peroxidation. During the lipid peroxidation reaction, lipid hydroperoxides are generated as primary products. Subsequent decomposition leads to the formation of reactive mediators including aldehydes, which can covalently modify biomolecules. We have recently found that lipid hydroperoxides, the primary peroxidative products, can universally react with primary amino groups to form *N*-acyl-type (amide linkage) adducts (10–15). In our previous studies, the formation of linoleic acid-derived lysine modification adducts, *N*^ε-(hexanoyl) lysine and *N*^ε-(azelaoyl) lysine, and DHA-derived adducts, *N*^ε-(succinyl) lysine and *N*^ε-(propanoyl) lysine, have been identified *in vitro* or *in vivo* by liquid chromatography-MS/MS or immunochemical analysis. In addition, the formation of *N*^ε-(hexanoyl) lysine also was detected, as well as *N*^ε-(glutaryl) lysine, during the reaction of oxidized AA with the lysine residue. The *N*-acyl-type adducts are specific to the peroxidation of polyunsaturated fatty acids; therefore, their formations are useful markers for the lipid peroxidation, protein modification, and related dysfunction that occur in these fatty acid-enriched tissues.

Dopamine is the endogenous neurotransmitter produced by nigral neurons. Dopamine loss can trigger not only prominent secondary morphological changes, such as density reduction of the dendritic spines, but also changes in the density and sensitivity of dopamine receptors (1); therefore, it is a sign of PD development. The reasons for dopamine loss are attributed to

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³ The abbreviations used are: PD, Parkinson disease; DHA, docosahexaenoic acid; AA, arachidonic acid; SUD, succinyl dopamine; PRD, propanoyl dopamine; HED, hexanoyl dopamine; GLD, glutaroyl dopamine; NOD, nonanoyl dopamine; LAD, lauroyl dopamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; PARP, poly(ADP-ribose) polymerase; 6-OHDA, 6-hydroperoxydopamine; ROS, reactive oxygen species; MS/MS, tandem mass spectrometry; HPLC, high performance liquid chromatography; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; DCF, dichlorofluorescein; DAT, dopamine transporter; NET, norepinephrine transporter; 5-HTT, serotonin transporter; HEDOPA, hexanoyl dihydroxyphenylalanine.

the molecular instability of dopamine. Some possible causes of dopamine loss are abnormalities of dopaminergic neurons (16), dopamine degradation by monoamine oxidase A (17) or auto-oxidation (18) and the reaction with amino acid cysteine (19). Dopamine is a member of catecholamine family. The catechol structure contributes to high oxidative activation of dopamine. Additionally, the N termini in the structure of dopamine may represent another reactive spot; however, little experimental evidence proves this. Based on our previously described reaction between lipid hydroperoxides and N-terminal residues, we focused on the possibility that reactive hydroperoxide species derived from lipid peroxidation may modify dopamine to form amide linkage dopamine adducts.

In the present study, we chemically synthesized four dopamine-modified adducts derived from DHA and AA. We were particularly interested in the formation of the dopamine adducts by chemical reactions and *in vivo* experiments, as well as the cytotoxicity evaluation using neuronal cells. All four dopamine adducts were detected upon incubation of dopamine with fatty acid hydroperoxides and an *in vivo* experiment using rat brain tissue. Furthermore, we focused on an AA-derived adduct HED, which induced severe cytotoxicity in human dopaminergic neuroblastoma SH-SY5Y cells compared with other adducts. The HED-induced cell death was found to include apoptosis that might be mediated by reactive oxygen species (ROS) and mitochondrial abnormality in SH-SY5Y cells. In addition, we found that the presence of monoamine transporters in the cells was essential for the HED-induced cytotoxicity, suggesting the specificity of the cytotoxicity to the cells. Taken together, the DHA- and AA-derived dopamine adducts may be useful biomarkers of the dopamine deficiency, and the formation of these adducts may indicate a novel mechanism responsible for the pathogenesis in Parkinson disease.

EXPERIMENTAL PROCEDURES

Materials—DHA, arachidonic acid, lipoxidase, GBR 12909 dihydrochloride, imipramine hydrochloride, *N*-acetyl-Asp-Glu-Aal-Asp-al, and RNase were obtained from Sigma-Aldrich (Tokyo, Japan). Dopamine-HCl was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Linoleic acid, hexanoic anhydride, glutaric anhydride, propanoic anhydride, succinic anhydride, lauric anhydride, and nonanoic anhydride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The antibodies against cytochrome *c* oxidase IV and poly(ADP-ribose) polymerase (PARP) were purchased from Cell Signaling Technology, Inc. (Boston, MA). Active caspase-3 rabbit monoclonal antibody was purchased from Epitomics, Inc. (Burlingame, CA).

Synthesis of *N*-Acyl Dopamine Adducts—The *N*-acyl dopamine adducts were chemically synthesized by incubating dopamine (0.5 mM) with carboxylic or methylester anhydride (0.5 mM) in 5 ml of 100 mM sodium phosphate buffer (pH 7.4)-saturated sodium acetate (1:1, v/v) for 60 min at room temperature. Succinic anhydride and propanoic anhydride were utilized for preparing the succinyl dopamine (SUD) and propanoyl dopamine (PRD), respectively; Hexanoic anhydride and glutaric anhydride were used for synthesizing the HED and glutaroyl dopamine (GLD), respectively. The synthesized adducts were purified by reverse phase HPLC using a Develosil ODS-HG-5

column (20 × 250 mm) in an isocratic system of 15 or 50% acetonitrile containing 0.1% trifluoroacetic acid at the flow rate of 6 ml/min. The elution profiles were monitored by absorbance at 280 nm. The amino residues in the dopamine adducts were identified by the ninhydrin reaction. The mass, structure, and formula of the synthesized molecule were identified by HPLC-MS, NMR, and electrospray ionization time-of-flight mass spectrometry analyses, respectively.

HPLC-Tandem Mass Spectrometry—The HPLC-MS/MS analysis was carried out using an API 2000 triple quadrupole mass spectrometer (Applied Biosystems) through a TurboIon-Spray source. Chromatography was carried out on a Develosil ODS-HG-3 column (2.0 × 250 mm) using an Agilent 1100 HPLC system. The chromatographic separation was performed by a gradient elution as follows: 0–10 min, linear gradient from 0.1% formic acid to 50% aqueous acetonitrile containing 0.1% formic acid; 10–15 min, hold; 15–20 min, linear gradient to 0.1% formic acid; flow rate = 0.2 ml/min. The instrument response was optimized by infusion experiments with the standard compounds using a syringe pump at the flow rate of 5 μ l/min. The dopamine adducts were detected using electrospray ionization MS/MS in the multiple reaction monitoring mode.

In Vitro Modification of Dopamine—DHA hydroperoxides were prepared from the DHA auto-oxidative reaction, and AA hydroperoxides were prepared using 15-lipoxygenase. The levels of lipid hydroperoxide were determined using a lipid hydroperoxide assay kit (Cayman Chemical Co., Ann Arbor, Michigan). Dopamine (2 mM) was incubated with 10 mM of DHA or AA hydroperoxides in phosphate buffer (pH 7.4) at 37 °C for different times. The reaction was terminated by immediate freezing at –80 °C. AA hydroperoxide was prepared as described previously (11).

In Vivo Detection of Dopamine Adducts—Brain homogenates of 7- and 27-week-old male F344/NSlc rats were used to detect the dopamine adducts. Briefly, the rat brain was removed and homogenized with phosphate-buffered saline containing 5% dibutylhydroxytoluene (5 mM) and EDTA (250 mM). After the addition of the deuterated dopamine adducts (20 μ M) as the internal standard, the homogenates were centrifuged at 3,000 rpm for 10 min. The pellet was then dissolved in 100 μ l of methanol. The detection was carried out by HPLC-MS/MS.

Cell Cultures and Drug Treatment Procedures—SH-SY5Y human dopaminergic neuroblastoma cells and NIH-3T3 mouse embryonic fibroblast cells were kindly donated by Dr. Maruyama (National Institute for Longevity Science). SH-SY5Y cells and NIH-3T3 cells were grown in Cosmedium-001 (Cosmo-Bio, Tokyo, Japan) containing 5% FBS and DMEM containing 10% FBS, respectively, and maintained at 37 °C in an atmosphere of 5% CO₂ in air. The 80% confluent cells were allowed to medium change with FBS-free DMEM overnight. The dissolved drugs were diluted by 1/500 or 1/1000 and added to fresh FBS-free DMEM to achieve the required concentration.

Assessment of Cell Viability—Cell viability was evaluated by an MTT assay. SH-SY5Y cells in 96-well plates were incubated with drugs for different times, followed by further incubation with 500 μ g/ml MTT at 37 °C for 2 h. Cell viability in some experiments was also measured using PI and Hoechst 33258 staining.

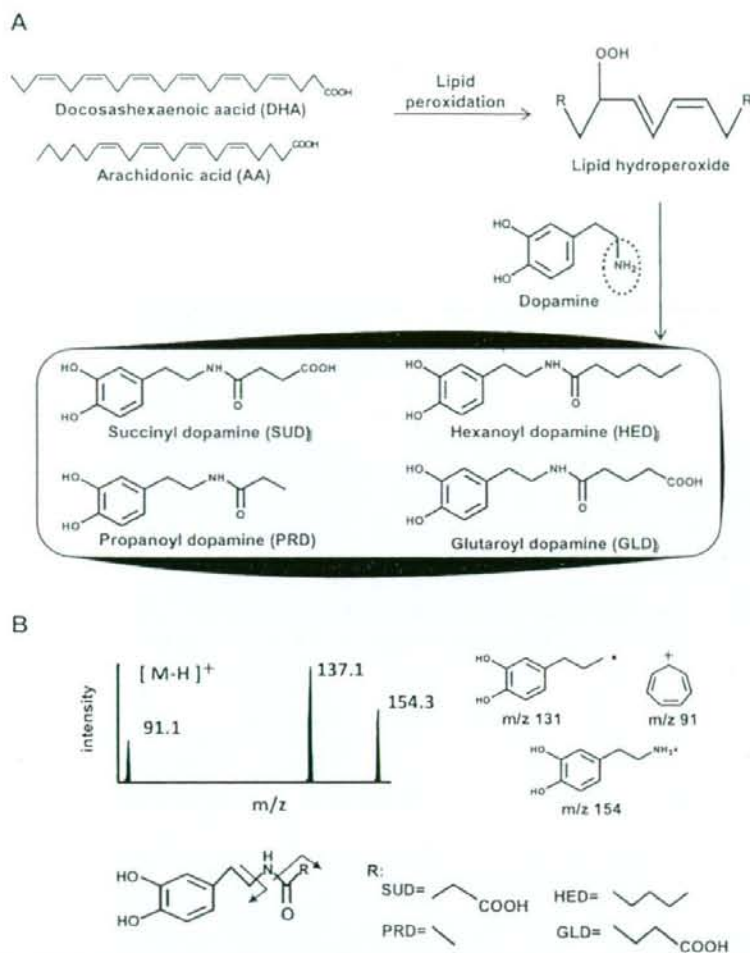


FIGURE 1. Proposed chemical formation scheme and HPLC-MS/MS analysis of DHA- and AA-derived dopamine adducts. *A*, proposed reaction scheme of DHA- and AA-derived dopamine adduct formation. *B*, the $[M-H]^+$ ion m/z 254, 210, 252, and 268 of SUD, PRD, HED, and GLD, respectively, were subjected to collision-induced dissociation, and the daughter ions were scanned (upper left panel). The proposed structures of individual ions are shown (upper right panel). The chemical structure composition of the dopamine adducts is proposed by fragmental analysis (lower panel).

ROS Measurement—Endogenous ROS level was detected by flow cytometry using $H_2DCF-DA$ (2',7',-dichlorodihydrofluorescein diacetate) (Molecular Probes). Briefly, the drug-treated cells were incubated with $H_2DCF-DA$ for 30 min, and the fluorescence of dichlorofluorescein (DCF) was measured using an EPICS Elite Flow Cytometer.

DNA Fragmentation Assay—The drug-treated SH-SY5Y cells were collected, suspended in 0.2 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 0.5% Triton X-100), and incubated at room temperature for 10 min. The samples were then centrifuged at $12,000 \times g$ for 10 min, and the supernatant containing the DNA cleavage products was incubated with 0.2 mg/ml proteinase K at $37^\circ C$ for 1 h followed by 0.1 mg/ml RNase A for 30 min at $50^\circ C$. The DNA fragments were purified by phenol/chloroform extraction and ethanol precipitation and

then separated on an ethidium bromide (0.5 mg/ml)-containing 2% agarose gel.

Subcellular Fraction of SH-SY5Y Cell—The cells are harvested by centrifugation at $600 \times g$ for 10 min, washed with phosphate-buffered saline, and resuspended with 5 volumes of Solution A (0.25 M sucrose, 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride). The cellular suspension was homogenized with a glass-glass homogenizer with 20 up and down passes of the pestle. The homogenate was then centrifuged at $750 \times g$ for 10 min. The resulting supernatant was collected and then centrifuged at $10,000 \times g$ for 15 min. The pellet was used as the mitochondrial fraction.

Western Blot Analysis—The cells were washed twice with phosphate-buffered saline, pH 7.0, and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 $\mu g/ml$ phenylmethylsulfonyl fluoride). After protein quantification, equal amounts of the protein (total protein, 20–50 μg) were boiled with Laemmli sample buffer for 5 min at $100^\circ C$. The samples were run on 10% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, incubated with 5% skim milk in TTBS (Tris-buffered saline containing 10% Tween 20) for blocking, washed, and treated with the primary antibodies. After washing with TTBS, the blots were further incubated for

1 h at room temperature with the IgG antibody coupled to horseradish peroxidase in TTBS. The blots were then washed three times in TTBS before visualization. An ECL kit was used for detection.

Statistical Analysis—All of the data were analyzed using Bonferroni/Dunn's multiple comparison procedure.

RESULTS

Chemical Formation of DHA- and AA-derived Dopamine Adducts—Based on the observations that lipid hydroperoxides, the primary products of lipid peroxidation, could universally react with primary amino groups to form *N*-acyl-type (amide linkage) adducts, and also within the chemical structure of dopamine, an amino residue is present, we chemically synthesized the four amide linkage dopamine adducts, SUD, PRD, HED,