Cyclophilin D 欠損マウスの確立は、Cyclophilin D依存的MPTが種々の疾患に関与するか否かを検討しうる有用な材料となっている。今回の検討からは、Cyclophilin D 依存的 MPT が mnd2 変異による運動神経変性疾患発症には大きく関与していないことが判明したが、アルツハイマー病ややパーキンソン病への関与の可能性もあり、これら疾患のモデルマウス系への利用も検討すべきであり、今後の重要な課題である。

iPLA2β欠損マウスは軸索変性を伴う神経異常を呈することが明らかになったが、ヒトの神経変性を伴う遺伝病であるINAD (infantile neuroaxonal dystrophy) や NBIA (Neuroaxonal degeneration with brain ion accumulation)においてiPLA2βに変異が見出されることから、我々の作製したiPLA2β欠損マウスは、INADやNBIAの病態解析や治療戦略の構築に貢献できるものと思われる。

E. 結論

MPTの分子メカニズムの解明と細胞死における役割を解明するために、MPTの構成成分でありその制御分子と考えられているCyclophilin Dの結合分子の探索を行い、Cyclophilin Dと相互作用する分子の存在を確認した。

MPTの関与が示唆されていたマウス運動神経変性疾患モデル MND2 マウスとの掛合わせを行い検討したが、シクロフィリン D の疾患発症への有意な関与は見出せなかった。

iPLA2β欠損マウスは軸索変性を伴う神経異常を呈することが明らかになり、ヒトの神経変性を伴う遺伝病である INAD (infantile neuroaxonal dystrophy) や NBIA (Neuroaxonal degeneration with brain ion accumulation)のマウスモデルとなることが分かった。

F. 健康危険情報

特になし

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H. 知的財産権の出願・登録状況
特　に　な　し

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Journal of Neural Transmission
Printed in Austria

Neuromelanin induces oxidative stress in mitochondria through release of iron: mechanism behind the inhibition of 26S proteasome

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Received May 15, 2005; accepted October 20, 2005
Published online December 16, 2005;

Summary. Parkinson's disease is characterized by the selective depletion of dopamine neurons in the substantia nigra, particular those containing neuromelanin. Involvement of neuromelanin in the pathogenesis may be either cytotoxic or protective. Recently we found that neuromelanin reduces the activity of 26S proteasome. In this paper, the detailed mechanisms behind the reduced activity were studied using neuromelanin isolated from the human brain. Neuromelanin increased the oxidative stress, but synthetic melanin did not. Superoxide dismutase and deferoxamine completely suppressed the increase, indicating that superoxide produced by an iron-mediated reaction plays a central role. Iron was shown to reduce *in situ* 26S proteasome activity in SH-SY5Y cells and the reduction was protected by antioxidants. These results suggest that iron released from neuromelanin increases oxidative stress in mitochondria, and then causes mitochondrial dysfunction and reduces proteasome function. The role of neuromelanin is discussed in relation to the selective vulnerability of dopamine neurons in Parkinson's disease.

Keywords: Neuromelanin, mitochondria, oxidative stress, Parkinson's disease, ubiquitin-proteasome system, ferrous iron.

Abbreviations

ACM 7-Aminomethylcoumarin; *DCF* 2′,7′-Dichlorofluorescein; *DFX* deferoxamine mesylate; *DMSO* dimethyl sulfoxide; *EGCG* (−)-epigallocatechin gallate; *GFP* green fluorescent protein; *H_2DCFDA* 2′,7′-dichlorodihydrofluorescein diacetate; *MEM* minimum essential medium; *MG132* *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; *NAC* *N*-acetyl cysteine; *NM* neuromelanin; *$NO^{\bullet}$* nitric oxide; *$O_2^{\bullet -}$* superoxide radical;

^{-}OH hydroxyl radical; *$ONOO^{-}$* peroxynitrite; *PBS* phosphate-buffered saline; *PD* Parkinson's disease; *PI* propidium iodide; *PSI* carbobenzoxy-L-isoleucyl-γ-*t*-butyl-L-glutamyl-L-alanyl-L-leucinal [Z-Ile-Glu(Obu^{t})-Ala-Leu-H (aldehyde)]; *SN* substantia nigra; *SOD* superoxide dismutase; *UP* ubiquitin-proteasome; *ZsGFP Zoanthus* sp. green fluorescence protein.

Introduction

In Parkinson's disease (PD), degeneration of dopamine neurons in the substantia nigra (SN) and accumulation of typical inclusion bodies, Lewy bodies, are characteristic pathological features. In PD, dopaminergic neurons of the SN and noradrenergic neurons of the locus coeruleus (LC) are preferentially affected (Hirsch et al., 1988). The presence of neuromelanin (NM) in these neurons suggests that NM may be involved in their vulnerability towards insults leading to cell death (Kastner et al., 1992). NM is synthesized from quinones and semiquinones produced by enzymatic or non-enzymatic oxidation of dopamine and noradrenaline in the SN and LC, respectively. The complex polymers contain also other oxidation metabolites of dopamine and L-DOPA, cysteinyl-DOPA (Odh et al., 1994), 5-S-cysteinyl-dopamine (Zhang and Dryhurst, 1994), proteinacious components and lipids (Gerlach et al., 1995; Zecca et al., 1994, 2000; Dzierzega-Lecznar et al., 2004; Fedorow et al., 2005a; Tribl et al., 2005). In addition, inorganic components, especially iron, copper and zinc are detected in NM (Bridelli et al., 1999).

The role of NM has been discussed either in a preventing or promoting way to the degeneration of nigral dopamine neurons. NM was reported to increase the vulnerability of SN neurons (Youdim et al., 1994; Offen et al., 1997), and an iron(III)-melanin complex was cytotoxic to dopaminergic neurons *in vitro* (Double et al., 2002). Increased production of reactive oxygen and nitrogen specifies (ROS, RNS) was proposed to account for the cytotoxicity of NM-metal conjugates (Ben-Shachar et al., 1991). More recently NM was reported to increase the secretion of cytokines and nitric oxide ($NO^{\bullet}$) from microglia, which might be also related to degeneration of dopamine neurons in PD (Wilms et al., 2003). On the other hand, NM has been proposed to be neuroprotective, by scavenging redox active metals (Fe, Cu and Mn), toxic metals (Cd, Hg and Pb) (Youdim et al., 1994; Zecca et al., 1994, 2002; Double et al., 2003) and other toxins, such as 1-methl-4-phenylpyrimidium ion (MPP^{+}) (D'Amato et al., 1986) and 1,2(*N*)-dimethyl-6,7-dihydroxyisoquinolinium ion, an oxidation product of *N*-methyl-(*R*)salsolinol, an endogenous dopaminergic neurotoxin (Naoi et al., 1994). In addition, NM synthesis reduces accumulation of cytotoxic quinone produced by dopamine oxidation (Sulzer et al., 2000).

In PD, oxidative stress, mitochondrial dysfunction, excitotoxicity and inflammation have been proposed to cause the cell death in nigro-striatal dopamine neurons. Mitochondrial dysfunction results in increased oxidative stress and on the other hand, ROS and RNS induce dysfunction in mitochondrial respiratory chain, suggesting that there is a complex interrelationship between the pathogenic factors. In addition, recent evidences suggest that failure of the ubiquitin-proteasome (UP) system leads to aggregation and accumulation of abnormal proteins to form the inclusion bodies and induce neuronal cell death (Leroy et al., 1998; McNaught et al., 2002a). The UP system is now considered to contribute to the etiopathogenesis of the sporadic form of PD, in addition to the familial forms (McNaught and Olanow, 2003b). Actually, the products of genes responsible for the familial parkinsonism, α-synuclein, ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), and parkin were found to co-localize with proteasomal subunits in Lewy body (Ii et al., 1997; Gai et al., 2000). "Aggresomes" formed in response to the

accumulation of abnormal proteins in neurons with reduced UP system was indicated as a precursor of Lewy body in PD (McNaught et al., 2002b).

Recently, we found that NM purified from the human reduced the activity of 26S proteasome (Shamoto-Nagai et al., 2004), both directly *in vitro* and also *in situ* and reduced the protein amounts of PA700 regulatory subunit. These results may be relevant with those found in the parkinsonian substantia nigra: the reduction in the activity of 20S proteasome and also the levels of the regulatory subunit, PA700 (19S, ATPase) and α-subunit of 20S proteasome (McNaught et al., 2003a). However, the detailed mechanism underlying the inhibition remains to be clarified.

In this paper, the effects of NM on the induction of oxidative stress were studied using mitochondria prepared from SH-SY5Y cells, and their effects on the *in situ* activity of proteasome were examined using SH-SY5Y cells transfected with a green fluorescent protein targeting to 26S proteasomes (proteasome sensor vector) (Shamoto-Nagai et al., 2003). Generation of hydroxyl radical ($^{\bullet}OH$), nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^{-}$) was quantitatively measured by use of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Crow, 1997). In addition, the effects of iron on the levels of oxidative modified protein, especially acrolein-protein conjugates were examined, the increase of which was confirmed in rotenone-induced UP system failure model (Shamoto-Nagai et al., 2003). The results are discussed in relation to the possible role of NM in the cell death in PD, especially about interaction among mitochondrial dysfunction, oxidative stress and deterioration of the UP system.

Materials and methods

Materials

2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was purchased from Molecular Probes (Eugene, OR, USA). Synthetic melanin was prepared by peroxidation of L-tyrosine with hydrogen peroxide. Deferoxamine mesylate (DFX), (−)-epigallocatechin gallate (EGCG) from green tea, and superoxide dismutase (SOD) from bovine erythrocytes were purchased from Sigma (St. Louis, MO, USA). Propidium iodide (PI) was purchased from Molecular Probes (Eugene, OR. USA). 2′,7′-Dichlorofluorescein (DCF), *N*-acetyl cysteine (NAC), L-cysteine and catalase from bovine liver, minimum essential medium (MEM) and other reagents were from Wako (Kyoto, Japan), and DL-α-tocopherol from Nacalai tesque (Kyoto, Japan). NO_2/NO_3 assay kit-F (fluorometric) was purchased from Dojindo (Kumamoto, Japan). Mouse anti-acrolein antibody was purchased from NOF (Tokyo, Japan). Inhibitors of proteasome, *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal (MG132) and benzyloxycarbonyl-L-isoleucyl-γ-*t*-butyl-L-glutamyl-L-alanyl-L-leucinal [PSI, Z-Ile-Glu(Obu^t)-Ala-Leu-H (aldehyde)] were purchased from Peptide Institute (Osaka, Japan). Human dopaminergic neuroblastoma SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented by 5% fetal calf serum in an atmosphere of 95% air and 5% CO_2.

Preparation of NM suspension

NM was isolated from the SN of neurologically normal adult individuals as previously described (Double et al., 2000). Brains were provided from the Austro-German Brain Bank in Würzburg. The use of post-mortem human brain tissue was approved by the Ethics Committee of the University Clinics of Würzburg. In brief, SN samples were homogenised in 20 ml water and centrifuged at 12 000 g for 10 min. The resulting pellets were washed twice with 50 mM phosphate buffer (pH 7.4), then incubated in 50 mM Tris buffer (pH 7.4) containing 0.5 mg · ml^{-1} SDS at 37°C for 3 h, followed by a further 3 h incubation with addition of 0.2 mg · ml^{-1} proteinase K in the same buffer. The pellets were pooled and consecutively washed with saline, water, methanol and hexane. Finally, the resulting dark pellet was incubated for three periods of 8 hrs each in 150 mM EDTA (pH 7.4) before being washed twice with water and dried under vacuum.

NM was dissolved in distilled water containing 15 mM L-cysteine and 10% dimethyl sulfoxide (DMSO) (L-Cyst-DMSO solution) to be 0.5 mg/ml in the final concentration. NM suspension was sonicated for 20 min and then shaken gently for 4 days for rehydration at room temperature under protection from light.

Isolation of mitochondria from SH-SY5Y cells

Mitochondria were prepared from SH-SY5Y cells according to Desagher et al. (1999). The cells were

gathered, washed with PBS and suspended in isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM HEPES, pH 7.5) supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Mannhein, Germany). The cells were homogenized for 20 strokes with a Dounce homogenizer, centrifuged at 500 g for 5 min, and the supernatant was centrifuged at 10 000 g for 30 min. The crude mitochondria were obtained as the pellet and suspended in PBS for following experiments.

Measurement of ROS-RNS with H_2DCFDA

The mitochondria were suspended in PBS and the production of ROS-RNS, $^{\bullet}OH$, $NO^{\bullet}$, and peroxynitrite ($ONOO^-$) produced from $NO^{\bullet}$ and superoxide ($O_2^{\bullet -}$), was quantified fluorometrically by measuring DCF produced from H_2DCFDA (Crow, 1997). H_2DCFDA was added to be 50 μM to the mitochondria suspension (20–30 μg protein in a total reaction volume of 1 ml PBS) in the presence or absence of NM suspension (1–5 μg/ml) or synthetic melanin (1–10 μg/ml) in dark at 37°C. The increase in DCF fluorescence at 504 nm with excitation at 520 nm was measured at every 30 min for 3 h in a RF-5000 spectrofluorometer (Shimadzu, Kyoto, Japan). The generation of ROS-RNS was expressed as mol DCF per min per mg protein. The effects of DFX, anti-oxidants and other agents on the increased ROS-RNS production by NM were also examined in the same way, after 15 min pre-incubation.

Assay for NO

NO is hydrolyzed into NO_2^- and NO_3^- in aqueous solution, and this assay is based on the measurement of NO_2^- with 2,3-diaminonaphthalene, which is produced by reduction of NO_3^- (Misco et al., 1993). Mitochondrial suspension (20 μg/ml) was incubated with or without NM or synthetic melanin (2.5 μg) in PBS and produced $NO^{\bullet}$ was measured according to the manufacture's instruction, and the increase in the fluorescence at 450 nm was followed with excitation at 365 nm. The amount of NO produced was quantified by comparison with the authentic NO standard, and expressed as nmol per min per mg protein.

Assay for the in situ activity of 26S proteasome, and apoptosis

To quantify the *in situ* proteasome activity, SH-SY5Y cells transfected with a proteasome sensor vector were prepared as described previously (Shamoto-Nagai et al., 2004). The vector was designed to express ZsGreen fluorescence protein (ZsGFP) fused to the degradation domain of mouse ornithine decarboxylase, a specific substrate for 26S proteasome. The reduction of 26S proteasome activity was assessed by measuring the ZsGFP fluorescence accumulated in SH-SY5Y cells transfected with the proteasome sensor vector (SH-PSV cells). SH-PSV cells were cultured in 6-well poly-L-lysine coated flasks in MEM with various concentration of iron with or without 25 μM DFX or antioxidants for 20 h. The living cells were gathered, then suspended in PBS(−). The fluorescence of the cell suspension at 505 nm with excitation at 493 nm was measured in a spectrofluorophotometer, RF-5300 (Shimadzu, Kyoto, Japan), and expressed as arbitrary fluorescence unit per mg protein. The protein amount was measured according to Bradford (1976).

At the same time SH-PSV cells were also subjected to fluorescence augmented flow cytometry (FACS) and the native fluorescence of ZsGFP was quantified. 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 and suspended in PBS and subjected to FACS analysis. Cells with lower DNA content showing PI staining less than G1 were defined to be apoptotic (subG1 peak) (Eckert et al., 2001).

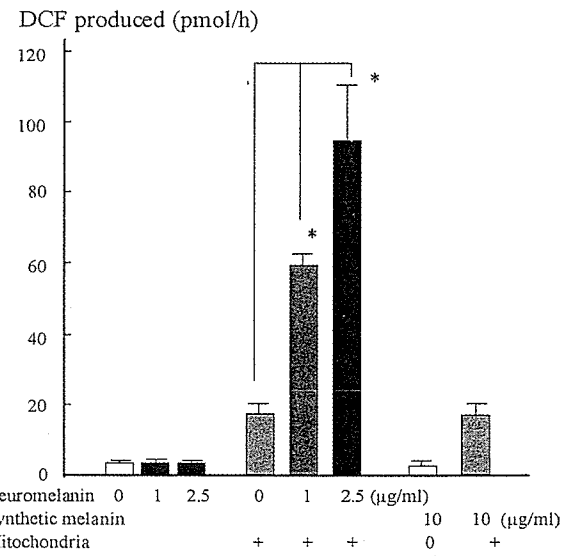

Fig. 1. ROS-RNS production in mitochondria. Mitochondria isolated from SH-SY5Y cells were incubated with or without NM or synthetic melanin. Generated ROS-RNS was quantitatively measured as DCF produced from H_2DCFDA and expressed as pmol/hr. The reaction mixture was composed of 1 ml PBS containing 0, 1, 2.5 μg human brain neuromelanin (NM) or 10 μg synthetic melanin in the presence or absence of mitochondria (28 μg protein). The column and bar represent the mean and SD of triplicate measurements of 3 independent experiments. $^*P<0.01$

Western blot analysis of acrolein-modified protein

SH-SY5Y cells were treated with 10–200 μM Fe^{2+} or Fe^{3+} in the absence or presence of 25 μM DFX for 20 h, gathered, washed with PBS, lyzed in Laemmli's sample buffer (100 mM Tris-HCl containing 4% SDS, 12% β-mercaptoethanol and 20% glycerol). The sample (20 μg protein/well) was separated by 10% polyacrylamide SDS-PAGE and blotted onto PVDF membrane, then, acrolein-modified proteins were visualized using antibody against anti-acrolein antibody, as reported (Shamoto-Nagai et al., 2003).

Statistics

Experiments were repeated at least 3 times. The data was 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

NM increased ROS-RNS production in mitochondria

The production of $^{\bullet}OH$, NO and $ONOO^-$ in mitochondria was quantified fluorometrically using DCF cleaved from H_2DCFDA as an indicator. As shown in Fig. 1, in the presence of mitochondria, NM increased DCF fluorescence in a dose-dependent way. NM alone did not increase DCF virtually at all. On the other hand, higher amount synthetic melanin did not increase ROS-RNS levels and even in the presence of mitochondria, the increase of DCF was not significant.

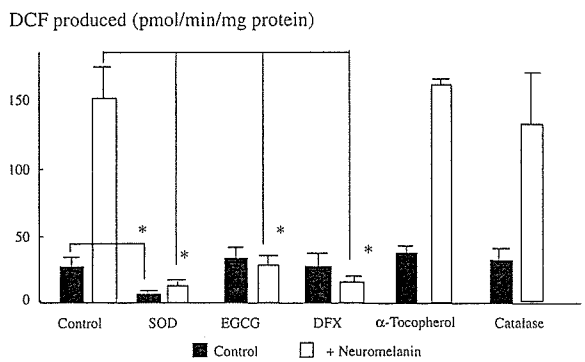

Fig. 2. Effects of SOD, (−)-epigallocatechin gallate (EGCG), deferoxamine mesylate (DFX), α-tocophenol and catalase on DCF production in mitochondria prepared from SH-SY5Y cells. Mitochondria (30 μg protein) were incubated with 2.5 μg/ml NM in PBS, and the effects of SOD (1000 units), EGCG (1 μM), DFX (1 μM), α-tocopherol (100 μM) and catalase (500 units) were examined. The column and bar represent the mean and SD of triplicate measurements of 3 independent experiments. $^*P<0.01$

Effects of DFX and anti-oxidants on ROS/RNS production by NM in mitochondria

The nature of ROS/RNS produced in mitochondria was studied by use of anti-oxidative enzymes and antioxidants. As shown in Fig. 2, SOD, but not catalase, reduced DCF production in mitochondria

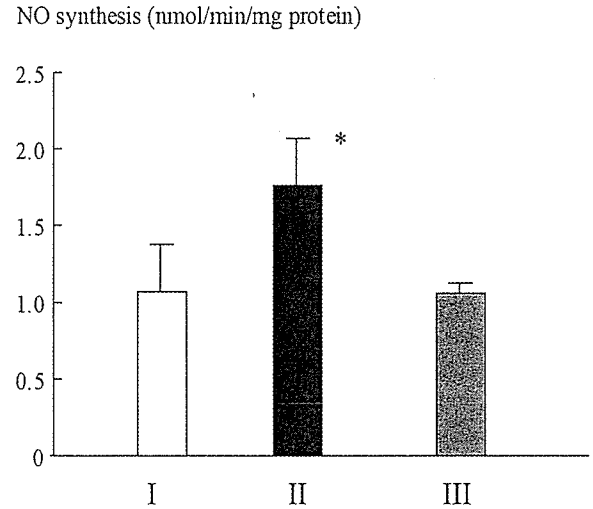

Fig. 3. Human brain neromelanin (NM) increased $NO^{\bullet}$ synthesis in mitochondria prepared from SH-SY5Y cells. NO_2^- and NO_3^- produced from $NO^{\bullet}$ were quantitatively measured with 2,3-diaminonaphthalene as an indicator, and expressed as nmol/min/mg protein. The amount of $NO^{\bullet}$ was calculated by comparing with $NO_2^{\bullet}$ standard. I: Mitochondria suspension (20 μg/ml) alone; II: mitochondria suspension incubated with NM (2.5 μg); III: mitochondria suspension incubated with synthetic melanin (2.5 μg). The column and bar represent the mean and SD of triplicate measurements of 3 independent experiments. $^*p<0.05$

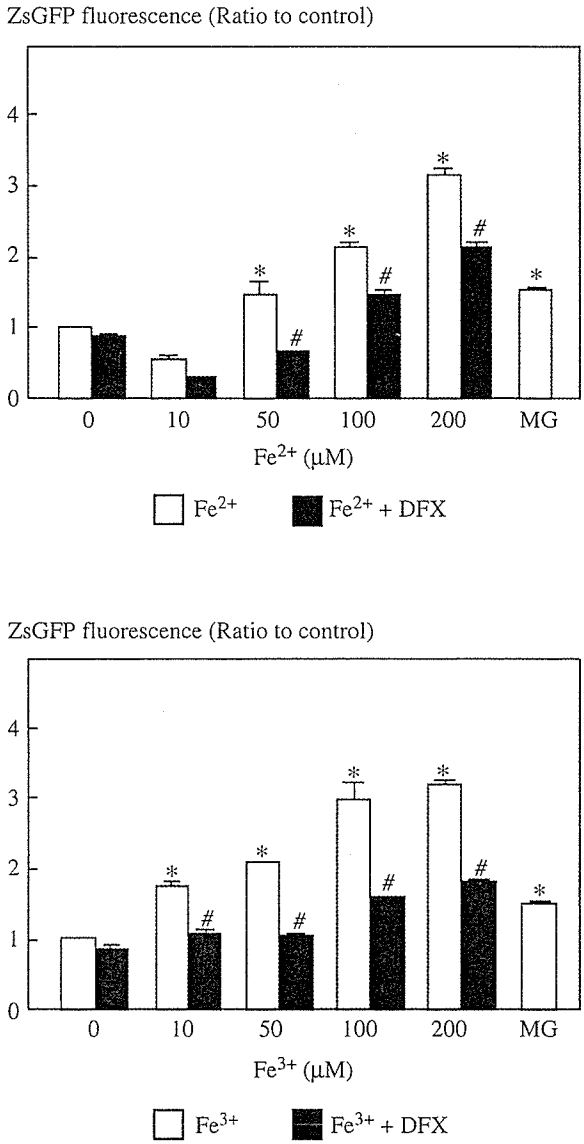

Fig. 4. Iron inhibited *in situ* activity of 26S proteasome in SH-SY5Y cells transfected with a proteasome sensor vector (SH-PSV cells). After the treatment with iron with or without deferoxamine mesylate (DFX) for 20 h, the fluorescence intensity of ZsGFP, which is coded by a proteasome sensor vetor, at 505 nm with excitation at 493 nm was quantified and expressed as arbitrary fluorescence unit/mg protein. The column and bar represent mean and SD of 3 experiments. After the treatment with iron, the fluorescence intensity increased in a dose-dependent manner. $^{*}p<0.05$ compared to the control. This increase was suppressed by 25 μM DFX significantly. $\#p<0.05$ compared to the cells treated with iron without DFX. MG; SH-PSV cells treated with 0.5 μM of MG132, a proteasome inhibitor, as a positive control

themselves. SOD, EGCG, and DFX significantly reduced the DCF production from mitochondira enhanced by NM. The reduction by SOD was most markedly, indicating that $O_2^{\bullet -}$ plays the key role in ROS-RNS production. EGCG, which preferentially scavenges NO (Akao et al., 2004), suppressed DCF production also. DFX was also potent to reduce DCF generation, suggesting the involvement of iron in the ROS-RNS production by NM. On the other hand, α-tocopherol did not affect DCF levels in mitochondria with or without NM.

Increased NO production by NM

The effects of NM and synthetic melanin on NO production were studied in mitochondria using 2,3-diaminonaphthalene as an indicator. As shown in Fig. 3, only NM increased NO level in mitochondria, whereas synthetic melanin did not affect the NO level in mitochondria.

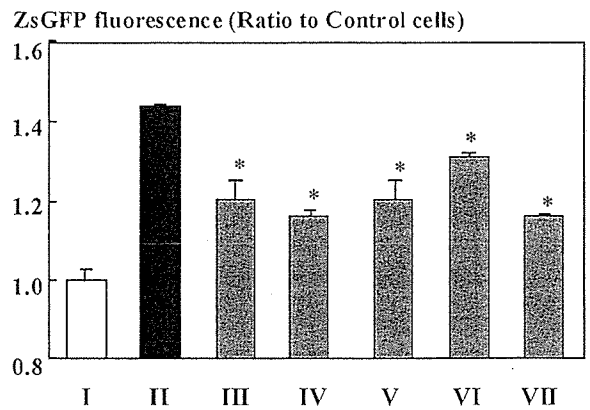

Fig. 5. Antioxidants suppressed the iron-induced inhibition of 26S proteasome activity in SH-SY5Y cells transfected with a proteasome sensor vector (SH-PSV cells). I: Control, II: treated with 100 μM Fe^{2+}, III–VII: pre-incubated with 50 μM ascorbic acid, 50 μM NAC, 0.5 μM (−)-epigallocatechin gallate (EGCG), 50 μM GSH and 50 μM of α-tocopherol, respectively for 30 min, then with Fe^{2+} for 20 h. The column and bar represent the mean and SD of 3 experiments. $^{*}p<0.05$ compared to the cells treated with Fe^{2+} alone

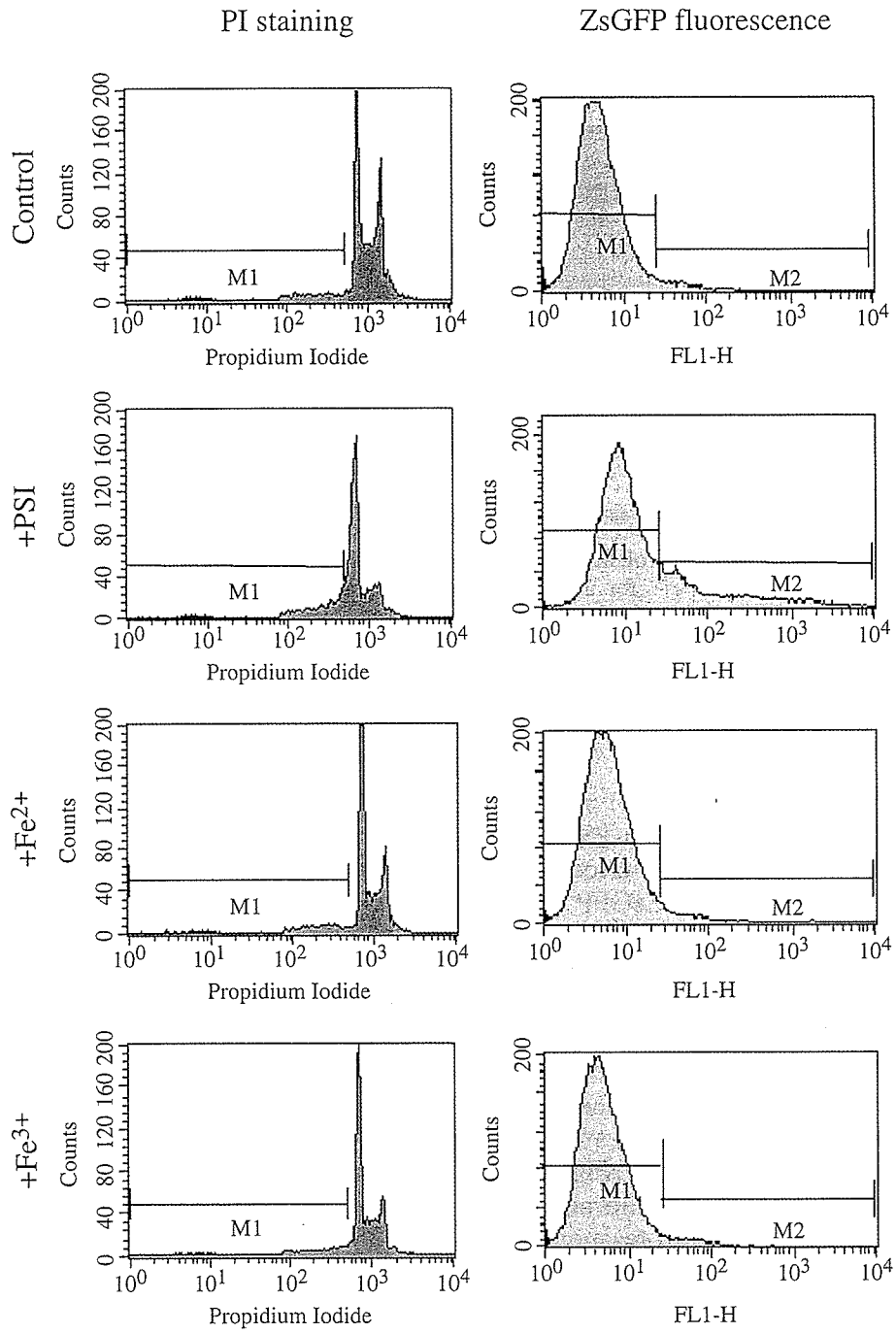

Fig. 6. The cytotoxicity of Fe^{2+} and Fe^{3+} on SH-SY5Y cells transfected with a proteasome sensor vector (SH-PSV cells). SH-PSV cells were cultured in 6-well poly-L-lysine coated flasks with 100 μM of Fe^{2+} or Fe^{3+} for 20 h. The cells were also subjected to FACS and the native fluorescence of ZsGFP, which is coded by proteasome sensor vector, was quantified. At the same time the cells were stained PI solution and subjected to FACS analysis to quantify the apoptotic cells. The number of cells with enhanced ZsGFP were 2.1% in non-treated cells and it increased to 3.1, 3.8 and 20.8%, by 100 μM of Fe^{2+} or Fe^{3+}, or 10 μM of PSI, proteasome inhibitor, respectively. At the same time, apoptotic cells increased from 4.8% in control to 9.6, 7.3 and 23.7% of the total in the presence of Fe^{2+}, Fe^{3+}, and PSI, respectively

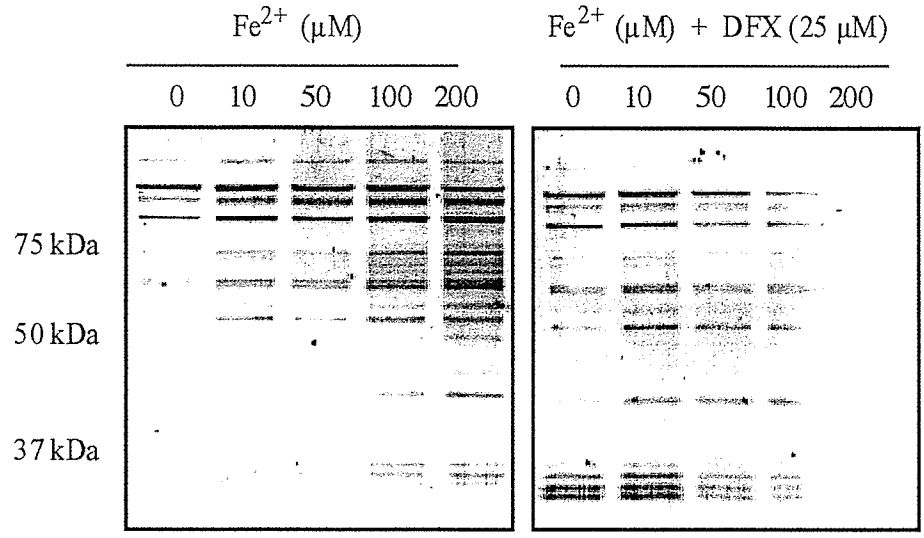

Fig. 7. Fe^{2+} treatment increased acrolein-modified protein in SH-SY5Y cells. SH-SY5Y cells were incubated with various concentrations of Fe^{2+} for 20 h, in the absence and presence of 25 μM deferoxamine mesylate (DFX). Acolein-modified protein was detected by Western blot analysis using antibody against acrolein-protein conjugates

Effect of iron on proteasome activity and viability in SH-SY5Y cells

The effects of iron (Fe^{2+} or Fe^{3+}) on the UP system were studied using SH-PSV cells. As shown in Fig. 4, after cultured in the presence of iron for 20 h, the ZsGFP fluorescence in SH-PSV cells increased in a dose-dependency way. Fe^{2+} and Fe^{3+} increased the accumulation of ZsGFP with almost the same intensity, and DFX (25 μM) suppressed the increase in ZsGFP fluorescence. Ascorbic acid, NAC, reduced glutathione (GSH), (−)-EGCG and α-tocopherol suppressed the accumulation of ZsGFP by Fe^{2+} (Fig. 5). On the other hand, Fe^{2+} and Fe^{3+} did not affect the *in vitro* activity of 20S proteasome samples prepared from SH-SY5Y cells (data not shown). These results show that iron inhibited the UP system, but it is not due to the direct inhibition of 20S proteasome enzymes.

The cytotoxicity of Fe^{2+} and Fe^{3+} was examined by fluorescence-augmented flow cytometry (FACS) after incubated with 100 μM Fe^{2+} and Fe^{3+} for 20 h, and the number of cells with enhanced ZsGFP increased to 3.1 and 3.4% of the total cells from 2.1% in control, whereas 10 μM PSI, a proteasome inhibitor, increased the cell number to 20.8%, as shown in Fig. 6. At the same time, apoptotic cells increased to 9.6 and 7.3% of the total cell number in the presence of Fe^{2+} and Fe^{3+}, and 23.7% in cells treated with PSI, respectively, from 4.8% in control.

Increased acrolein-modified protein by iron treatment

To estimate the effects of iron on lipid peroxidation, acrolein, one of the most reactive aldehyde, was detected with antibody against acrolein-conjugated protein. As shown Fig. 7, acrolein-adducted protein increased in the cells treated with Fe^{2+}, in a dose-dependent way. DFX reduced the amount of acrolein-modified protein significantly.

Discussion

These results clearly demonstrate that NM, in contrast to synthetic dopamine melanin without iron, increases formation of ROS-RNS and induces onset of oxidative stress in mitochondria. In this case, it should be empha-

sized that mitochondria are essentially required for the generation of ROS-RNS. In mitochondria prepared from dopaminergic SH-SY5Y cells, ROS is produced from $O_2^{\bullet -}$ via oxidative phosphorylation, while H_2O_2, at least in part, is generated *via* oxidation of dopamine by monoamine oxidase. About RNS recent reports suggest the existence of nitric oxide synthase in mitochondria (Elfering et al., 2002). In our system using DCF, measurable ROS-RNS species were characterized as $^{\bullet}OH$, $NO^{\bullet}$ and $ONOO^-$, which is produced by a reaction of $NO^{\bullet}$ with $O_2^{\bullet -}$ (Crow, 1997). DFX, an iron chelator, markedly reduced ROS-RNS production, suggesting that iron released form NM catalytically increases these kind of ROS-RNS production in mitochondria. Indeed, iron is well known to increase $^{\bullet}OH$ synthesis from H_2O_2 by the Fenton reaction, or by the Haber-Weiss reaction by the reaction of $O_2^{\bullet -}$ and H_2O_2 (Halliwell, 1992). In the system used in this paper SOD can completely suppresses the increase in DCF by NM, but catalase did not, indicating that the role of H_2O_2 is negligible. On the other hand, we showed that NM increases NO production by mitochondria. These results indicate that NM increased $O_2^{\bullet -}$ and NO simultaneously in mitochondria and produced $ONOO^-$, which is a powerful oxidant that may decompose further to the very reactive $^{\bullet}OH$. It is consistent with our previous data that in SH-SY5Y cells, RNS is the major source of DCF production (Maruyama et al., 2001).

The function of NM has yet to be established, but it is considered as an endogenous iron-binding molecule in pigmented neurons (reviewed in Fedorow et al., 2005b). It may therefore play a physiological role in intraneuronal iron homeostasis. Support for this theory comes from changes in NM in the PD brain where significantly less iron is bound to NM than that seen in the normal brain (Lopiano et al., 1990). This suggests that changes in iron-binding to NM result in increased levels of intraneuronal free iron and the subsequent cell damage observed in PD. Isolated human NM consists of 2.8% iron as estimated by Mössbauer spectroscopy (Gerlach et al., 1995), while the concentration of Fe^{3+} in the SN has been estimated using electron paramagnetic resonance at 6780 ng iron/mg intact SN tissue or 11300 ng iron/mg isolated NM (Shima et al., 1997). Iron binding studies using NM isolated from the human SN demonstrated that NM contains high ($K_d = 7.18 \pm 1.08$ nM) and low-affinity binding sites ($K_d = 94.31 \pm 6.55$ nM) for Fe^{3+} (Double et al., 2003). Our recent data demonstrates that a purely Fe^{3+} signal can be measured from intact frozen SN tissue using Mössbauer spectroscopy (Double et al., 2003). The interaction of iron with NM is of interest because the behaviour of NM changes in the presence of iron; instead of inactivating free radicals, it begins to act as an effective pro-oxidant. Recent *in vitro* studies suggest that iron is releasable under certain circumstances from NM to interact in free radical-producing pathways (Double et al., 1999): In the absence of iron, isolated human NM significantly decreased membranal damage in rat cortical homogenates *in vitro* as measured by lipid peroxidation. Further when NM was added together with iron the amount of lipid peroxidation measured was significantly less than that induced by iron alone. These results support the hypothesis that NM has antioxidant properties and can protect the cell from radical-induced damage. In contrast, when iron-saturated NM was added to the membrane homogenate, cell damage was significantly increased to 264% of that induced by NM alone; this damage was significantly attenuated by the addition of the iron chelator DFX (Double et al., 1999). These data were relevant with the result that iron increased lipid peroxidation product, acrolein-modified proteins. These and the previous results support the hypothesis that NM can have a protective influence on the cell, but can be

detrimental when iron levels rise above a certain level.

Recently we found that mitochondrial dysfunction caused by rotenone, a complex I inhibitor, increased protein modification by acrolein, and reduced the activity of proteasome, through binding of aggregated oxidized protein to the catalytic site of 20S proteasome and direct adduction of acrolein to 20S protosome itself (Shamoto-Nagai et al., 2003). More recently we found that NM inhibited the *in vitro* and *in situ* activity of 26S proteasome in SH-SY5Y cells (Shamoto-Nagai et al., 2004). The results reported here clearly show that NM is a source of cytotoxic iron and induces the onset of oxidative stress in mitochondria leading to the dysfunction. As the result, decreased ATP synthesis and enhanced oxidative stress may induce the deterioration of UP system with increased oxidative-modified proteins, such as acrolein-conjugated ones. The capacity of NM to regulate iron level in the cells may determine the vulnerability of dopamine neurons in aging and PD by initiating the malignant cycle between the mitochondrial dysfunction, increased oxidative stress and impairment of the UP system.

Acknowledgements

This work was supported by a Grant-in-Aid on Scientific Research for Young Scientists (B) for M. S. and (C) (W. M.) and (A) (W. M. and T. O.) from Japan Society for the Promotion of Science, Grant for Research on Dementia and Bone Fracture (W. M., M. N.) from the Ministry of Health, Labor and Welfare, Japan. P.R. was supported by the National Parkinson Foundation, Miami, Florida, USA.

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