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Endogenous dopamine (DA) renders dopaminergic cells vulnerable to challenge of proteasome inhibitor MG132

ZHIDONG ZHOU, SZEYEN KERK, & TIT MENG LIM

Department of Biological Science, National University of Singapore, 14 Science Drive 4, Singapore 117543

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Abstract

This study demonstrated that dopaminergic MN9D and PC12 cells were more vulnerable than non-dopaminergic N2A cells to the challenge by proteasome inhibitor MG132, which could be alleviated by reductants and alpha-methyl tyrosine (alpha-MT), a specific tyrosine hydroxylase inhibitor. Furthermore, challenging non-dopaminergic N2A cells with exogenous DA could aggravate MG132-induced cell viability decrease, which could be abrogated by reductants but not by alpha-MT. It was observed that alpha-MT could decrease endogenous DA content in dopaminergic MN9D and PC12 cells while N2A cells could take in exogenous DA into cytosol. The endogenous DA in dopaminergic cells was demonstrated to inhibit proteasome activity in the cells and further sensitize the proteasome to MG132 inhibition. In addition, the endogenous DA was also implicated for the increased level of lipid peroxidation and ubiquitinated proteins as well as inclusion bodies formation when non-dopaminergic cells were challenged with exogenous DA. Taken together it is proposed that endogenous DA in dopaminergic neurons could promote selective dopaminergic neurodegeneration, especially under the conditions of exopathic or idiopathic defects of ubiquitin–proteasome system (UPS), which may be abolished by reductant remedy.

Keywords: *Alpha-methyl tyrosine, dopamine, dopaminergic neurodegeneration, Parkinson's disease, proteasome inhibitor MG132, reductants*

Introduction

Parkinson's disease (PD) was first described by James Parkinson in 1817. It is the second most common progressive neurodegenerative disease after Alzheimer's disease, affecting $\sim 1\%$ of the population over age 60 [1]. The mechanisms underlying the selective and progressive loss of nigral dopaminergic neurons in PD are still unclear. Several lines of recently generated evidence suggest that dysfunction of ubiquitin–proteasome system (UPS) is one of the causal factors of PD and the defects in UPS has been proposed to be a universal point underlying both the familial and sporadic forms of PD [2]. The UPS is the primary proteolytic complex responsible for the elimination of unwanted and misfolded intracellular proteins [3]. Impairment of the UPS was found to lead to dopaminergic cell death and inclusion body formations in both cell culture and animal models of PD [4].

On the other hand, dopamine (DA) related oxidative stress was suggested to be the key event in the genesis of PD [5]. DA oxidation could produce reactive oxygen species (ROS) and toxic DA quinones related to neuron degeneration in PD [6]. Furthermore complicated interaction and cooperative effect between malfunction of UPS and DA contributing to PD have been documented [7–11]. DA has been found to induce proteasome inhibition [7] or to aggravate proteasome inhibition leading to cytotoxicity [8]. DA was found to enhance the formation of

Correspondence: T. M. Lim, Department of Biological Science, National University of Singapore, 14 Science Drive 4, Singapore 117543. Tel: 65-65163334. Fax: 65-67792486. Email: dbsltm@nus.edu.sg

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aggregates under proteasome inhibition in PC12 cells [9]. Inhibition of proteasome activity was found to sensitize DA neurons to protein alterations and oxidative stress [10]. The application of the proteasome inhibitor could increase the toxicity of DA and the levels of protein oxidation following administration of DA [11]. Therefore UPS defects might cooperate with the endogenous DA in the cells leading to selective dopaminergic neurodegeneration in PD.

However, some recent *in vivo* studies yielded inconsistent results regarding effects of proteasome inhibitors on dopaminergic neurodegeneration [12]. The proteasome inhibition was found to cause nigral degeneration with inclusion bodies in rats [13], but similar works using the same protocol failed to observe any alterations in locomotor activity or striatal DA and its metabolites [12]. Furthermore, the proteasome inhibitors were found to have some protective effect on dopaminergic neurons [14]. Therefore, the relationship between UPS and the pathogenesis of PD may be more complex than envisaged. In this study, we demonstrated, for the first time, that it was the endogenous DA in MN9D and PC12 cells that rendered dopaminergic cells vulnerable to challenge by proteasome inhibitor MG132. The endogenous DA was implicated to induce proteasome inhibition and sensitize proteasome to MG132 inhibition as well as to increase levels of lipid peroxidation and ubiquitinated proteins. Our findings support the hypothesis that cooperative effects between UPS malfunction and endogenous DA in cells may contribute to selective dopaminergic neurodegeneration.

Materials and methods

Chemicals

Ascorbic acid (AA), dopamine (DA), Alpha-methyl tyrosine (alpha-MT), proteasome inhibitor MG132 (dissolved in dimethyl sulphoxide), the fluorogenic substrate (succinyl-leu-leu-val-try-7-amido-4-methyl-coumarin) for proteasome activity detection, glutathione (GSH) and sodium tyrosinase (from mushroom) were all purchased from Sigma (St. Louis, USA).

Cell lines

Dopaminergic neuronal MN9D [15], PC12 cells and non-dopaminergic neuronal N2A cells (all derived from mice) were cultured and maintained in DMEM with 10% (v/v) foetal bovine serum (FBS) (Clonetech, CA, USA) and 1% (v/v) penicillin-streptomycin (Sigma, USA). To suppress DA synthesis in the cells, MN9D, PC12 and N2A cells were cultured and maintained in DMEM in the presence of 2 mm alpha-MT (a specific tyrosine hydroxylase inhibitor). Cells were maintained in alpha-MT containing DMEM for at least one passage before they were used for MG132 toxicity study.

Assay of malondialdehyde (MDA)

To detect the extents of lipid peroxidation in cells related to endogenous DA, content of MDA was measured according to Ledwozyw et al. [16] method. Cells were harvested and suspended in PBS, then disrupted by sonication (Sonics VCX 130, USA). After sonication, cell sample was centrifuged at 2000 g for 15 min to obtain the homogenate supernatant. MDA content was estimated by the thiobarbituric acid reactions as follows: 0.5 ml of supernatant was mixed with 2.5 ml 1.22 m trichloroacetic acid in 0.6 m HCl and allowed to stand for 15 min. Then 1.5 ml thiobarbituric acid (TBA) solution was added (TBA solution was obtained by dissolving 500 mg of TBA in 6 ml 1 m NaOH and then adding 69 ml H_2O) and thereafter heating for 30 min in a boiling water bath. Then the mixture was cooled to room temperature and 4 ml of n-butanol was added to it and the mixture was vigorously shaken for 3 min and centrifuged for 10 min at 1500 g. After that, the organic layer was removed and its absorbance at 532 nm against n-butanol was measured. The concentration of MDA in the samples was determined from the standard curve plotted by using malondialdehyde bis-methyloacethal.

MTT assay

Cell viability was checked by MTT analysis. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Duchefa, Netherlands) was prepared as 2 mg/ml stock solution in PBS and stored at 4°C. The MTT/DMEM solution [15% (v/v) of 2 mg/ml MTT stock solution mixed with 85% (v/v) DMEM] was prepared freshly. Cells in 24-well dishes were washed with PBS and incubated in 500 µl of MTT/DMEM solution in the dark at 37°C for 3 h. The solution was then aspirated without disrupting the cells and 500 µl of solubilizing solution [0.04 m hydrochloric acid/isopropanol plus 3% (w/v) SDS] was added and mixed well. The plates were incubated at room temperature for 1 h in the dark. The solution optical density (OD) was measured at 595 nm using a spectrophotometer (SpectraMAX 250 with software SOFTmax PRO 4.3 LS. USA) in a 96-well plate (Iwaki, Japan).

Preparation of cell extract for proteasome activity detection

Cell extracts were prepared after DA, reductants or alpha-MT treatment. Cells were washed three times with PBS and suspended in 100 µl Hepes buffer (5 mm Hepes, 1 mm EDTA, pH 7.5) and then disrupted by sonication (Sonics VCX 130, USA) and

centrifuged at 18 000 g for 15 min. The supernatant was used for proteasome activity detection.

Proteasome activity detection

Proteasome chymotrypsin peptidase activity was determined by procedures as previously reported [17]. For each sample, 5 μl cellular extract (15 μg protein) was added to one well of a 96-well plate (black colour, NUNCLON™ Δ Surface, Nalge Nunc International, Denmark) containing 250 μl of buffer A (20 mm Hepes, 0.5 mm EDTA, 0.035% (w/v) SDS, pH 8.0). Subsequently, 5 μl of solution containing MG-132 were added and incubated at 37°C for 30 min, then 5 μl of fluorogenic substrate (750 μl acetonitrile, 249 μl sterile distilled water, 1 μl trifluoroacetic acid, 1 mg fluorogenic substrate (succinyl-leu-leu-val-try-7-amido-4-methylcoumarin, Sigma, USA)) was added and further incubated at 37°C for 30 min. Substrate hydrolysis was determined by measuring fluorescence of liberated 7-amido-4-methyl coumarin by a fluorometer at 380 nm excitation wavelength and 440 nm emission wavelengths.

Detection of DA content in cells

To detect DA content in MN9D and PC12 cells in the presence or absence of 2 mm alpha-MT, cells were cultured in DMEM containing 2 mm alpha-MT for one passage, then cells were harvested and suspended in 400 μl 0.5 m perchloric acid, sonicated for 1 min (pulse on: 5 s, pulse off: 3 s, amplitude: 40%), centrifuged at 20 000 g 20 min at 4°C and analysed by HPLC procedure. To detect DA content in PC12 and N2A cells with or without DA challenge, $\sim 2 \times 10^7$ PC12 and N2A cells were treated with or without 800 μm DA in DMEM for 0–3 h. Then cells were harvested and analysed by HPLC as above-mentioned.

Improved HPLC procedure

A new HPLC procedure was performed using AKTA purifier HPLC system (Amersham, Sweden) with a UV detector (detection wave length was set as 280 nm) as well as a reversed-phase column (DENALI™ Monomeric RPC C18 4.6 mm × 250 mm, 120 Å Pore Size VYDAC 238DE™ Series, Grace Vydac, USA) and analysed under the control of UNICON 4.11 program. The new HPLC procedure was set up and performed as follows: the flow rate was 1 ml/min; the column was first equilibrated with 1 column volume (CV) elution buffer A (8% methanol (v/v), 0.1 m sodium phosphate, 20 mm sodium 1-heptane-sulphonate, 0.1 mm EDTA. Adjust to pH 4.75 with NaOH). After that samples were loaded (100 μl) and empty loop with 200 μl elution buffer A, followed by 0.5 CV elution buffer A. Then gradient wash down with gradually increased mixing percentage (volume of elution buffer B/volume of column wash (%)) of elution buffer B (80% (v/v) acetonitrile in distilled water) began. The start concentration of elution buffer B was 0% (v/v) and the target concentration of elution buffer B was 30% (v/v) with the length of gradient of 3 CV and gradient delay of 5 CV. All solutions for HPLC analysis were filtered through 22 μm membranes and degassed before use.

Western blot analysis

Cells were collected in lysis buffer (100 mm HEPES pH 7.5, 5 mm magnesium chloride, 150 mm sodium chloride, 1 mm EDTA, 1% v/v Triton and 1% v/v protease inhibitor cocktail (Calbiochem, Germany)) with the help of a cell scraper and centrifuged at 12 000 g at 4°C for 10 min. From the supernatant, 50 μg proteins were resolved by NuPAGE® 10% Bis-Tris Gel, 1.0 mm (Invitrogen, USA). The proteins were transferred onto a nitrocellulose membrane using an electric blotting apparatus (Biorad, USA), blocked with StartingBlock™ (PBS) Blocking buffer (Invitrogen, USA) for 30 min at room temperature before incubation with primary anti-body (mouse monoclonal anti-ubiquitin antibody, sc-58448, Santa Cruz Biotechnology, USA 1:100) in the same blocking buffer overnight at 4°C. The membrane was washed 5 × 5 min each with washing buffer A (150 mm sodium chloride plus 13 mm Tris-hydrochloric acid pH 7.5 and 0.1% v/v Tween 20) and subsequently incubated with the secondary antibody (goat anti-mouse IgG HRP, sc-2031, Santa Cruz Biotechnology, USA, 1:2000) in blocking buffer for 1 h at room temperature. Following subsequent washes the blots were developed with the enhanced chemiluminescent kit (Pierce, USA) on Kodak CL-Xposure™ films.

Immunochemistry

Immunochemistry was performed according to the method of Pardo et al. [18]. Briefly, after overnight DA challenge, N2A cells was washed with PBS and then fixed with freshly prepared 4% (w/v) paraformaldhyde in PBS at room temperature for 1 h. After PBS wash for 10 min, cells were blocked with blocking buffer (5% goat serum, 5% horse serum in PBS) for 30 min and then treated with primary antibody (mouse monoclonal anti-ubiquitin antibody, sc-58448, Santa Cruz Biotechnology, USA 1:20) in blocking buffer overnight at 4°C. The cells were washed with PBS three times (5 min each) then treated with second antibody (Biotin anti-mouse IgG1 (A85-1), cat No: 553441, Pharmingen, USA 1:100) in blocking buffer at room temperature for 1 h. After PBS wash three times (5 min each), cells were further treated with Streptavidin/HRP (P0397,

Dako, Denmark 1:200) for 30 min. After PBS wash three times (5 min each), cells were developed with 0.05% (w/v) DAB solution in PBS with 0.015% (w/v) H_2O_2 and observed under microscopy.

Data analysis

Statistical analyses were conducted using one- or two-way ANOVA followed by *post hoc* Student's *t*-test using software Minitab 14. Graphs were constructed with SigmaPlot 2001 software.

Results

Dopaminergic cells are more sensitive to toxicity of proteasome inhibitor MG132, which could be alleviated by reductants

We first verified the deteriorative effect of proteasome inhibition by proteasome inhibitor MG132 on cell viability of dopaminergic MN9D and PC12 cells as well as non-dopaminergic N2A cells. Challenging two dopaminergic cell lines (MN9D, PC12 cells) and non-dopaminergic N2A cells with proteasome inhibitor MG132 overnight caused significant cell viability decrease in a MG132 dosage dependent manner (Figure 1). The dimethyl sulphoxide (DMSO), solvent for MG132, had no influence on cell viability changes in MN9D, PC12 and N2A cells (data not shown). However, dopaminergic MN9D cells and PC12 cells were more sensitive to MG132-induced cell death, compared with non-dopaminergic N2A cells (Figure 1). Incubation of 0.5 μm MG132 overnight could decrease the cell viability of MN9D and PC12 cells to ~50% of control level, while similar extent of cell viability decrease of N2A cells could be attained by overnight challenge of MG132 at a high dosage of 2.0 μm (Figure 1). Furthermore the cell viability decrease of dopaminergic MN9D and PC12 cells induced by MG132 challenge could be significantly alleviated when the challenge of MG132 was in the presence of reductants (100 μm GSH, 1 mm Vitamin E and 400 μm AA, respectively) (Figure 1A–F). On the other hand, the same concentration of reductant treatment failed to alleviate the non-dopaminergic N2A cell viability decrease caused by MG132 challenge (Figure 1G–I), although 1 mm Vitamin E had only a slight elevating effect (Figure 1I).

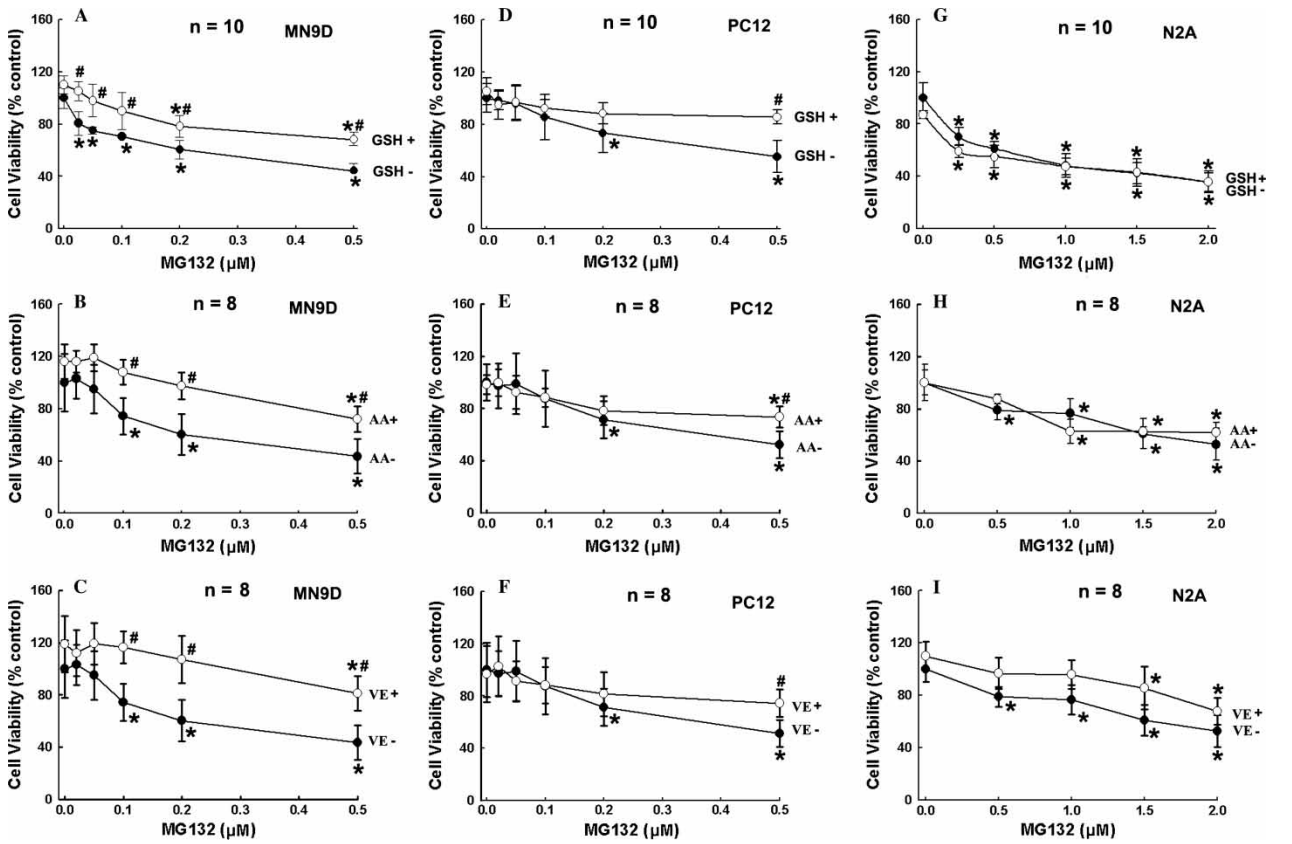

Figure 1. Cell viability decrease in dopaminergic but not in non-dopaminergic cells induced by proteasome inhibitor MG132 could be ameliorated by GSH, Vitamin E and AA. Dopaminergic MN9D (A–C) and PC12 (D–F) cells and non-dopaminergic N2A (G–I). Cells were challenged with MG132 overnight with (+) or without (−) 400 μM AA, 1 mm Vitamin E (VE), 100 μM GSH or 2 mm alpha-MT (MT). Cells without challenge were as 'controls'. Viability of cells were detected by MTT assay and shown as '% control'. *at least $p < 0.05$, compared with controls. # at least $p < 0.05$, compared with cells challenged with same concentration of MG132 only.

The high sensitivity of dopaminergic cells to proteasome inhibitor MG132 could also be alleviated by alpha-MT, a specific tyrosine hydroxylase inhibitor

Next, we demonstrated that the cell viability decrease of dopaminergic MN9D and PC12 cells induced by MG132 challenge could be significantly alleviated when the challenge was in the presence of 2 mm alpha-MT (Figure 2A and B) The alpha-MT is a specific tyrosine hydroxylase (TH) inhibitor to inhibit endogenous DA production, because TH is the key enzyme for DA synthesis in dopaminergic cells. However the same concentration of alpha-MT treatment failed to alleviate the cell viability decrease of non-dopaminergic N2A cells induced by overnight challenge of MG132 (Figure 2C). Therefore, the endogenous DA in MN9D and PC12 cells seemed to be an important factor responsible for aggravated dopaminergic cell viability decrease under proteasome inhibitor MG132 challenge.

Challenge of non-dopaminergic N2A cells with exogenous DA could sensitize N2A cells to toxicity of MG132, which could be alleviated by reductants but not by alpha-MT

We then challenged non-dopaminergic N2A cells with 50 μm exogenous DA alone overnight and this treatment could not induce significant cell viability decrease of N2A cells (Figure 3). However, when N2A cells were challenged with 50 μm DA together with 0–2 μm MG132 overnight there was a more dramatic cell viability decrease in a MG132 dosage dependent manner, compared with the cell viability decrease challenged with 0–2 μm MG132 only overnight (Figure 3). We further demonstrated that such dramatic cell viability decrease induced by MG132 plus DA could be significantly ameliorated by incubation of N2A cells simultaneously with 1 mm Vitamin E (Figure 3A), 400 μm AA (Figure 3B) or 100 μm GSH (Figure 3C). However, the cell viability decreases of N2A cells induced by the combined challenge of 0–2 μm MG132 and 50 μm exogenous DA overnight could not be alleviated by alpha-MT (Figure 3D).

Alpha-MT could significantly decrease DA content in dopaminergic cells

Our HPLC study disclosed that dopaminergic MN9D and PC12 cells contain endogenous DA in cells and culture of dopaminergic cells in the presence of 2 mm alpha-MT for at least one passage could significantly decrease DA contents in dopaminergic MN9D and PC12 cells (Figure 4A).

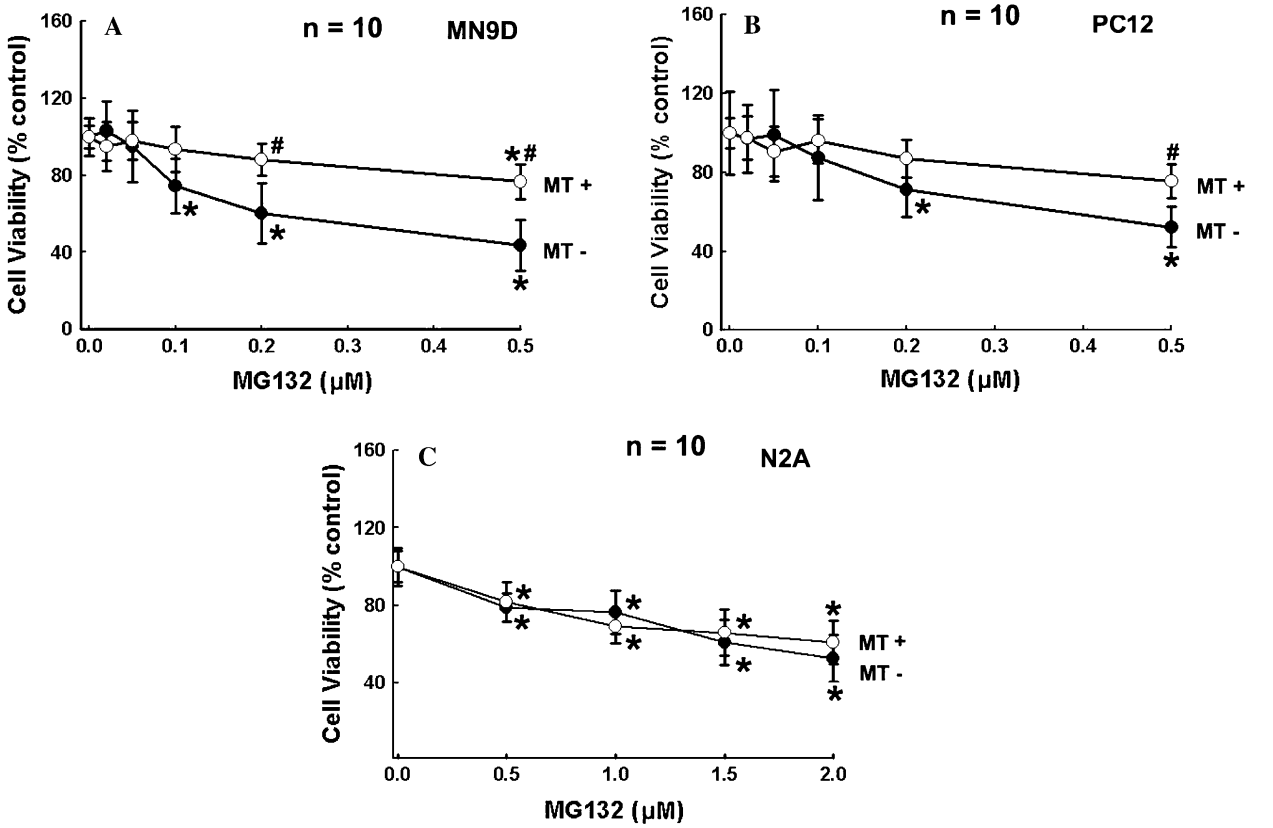

Figure 2. Cell viability decrease in dopaminergic but not in non-dopaminergic cells induced by proteasome inhibitor MG132 could be ameliorated by TH inhibitor, alpha-MT (MT). Dopaminergic MN9D (A) and PC12 (B) cells and non-dopaminergic N2A (C) cells were challenged with MG132 overnight with (+) or without (−) 2 mm alpha-MT (MT). Cells without challenge were as 'controls'. Viability of cells were detected by MTT assay and shown as '% control'. *at least $p < 0.05$, compared with controls. # at least $p < 0.05$, compared with cells challenged with same concentration of MG132 only.

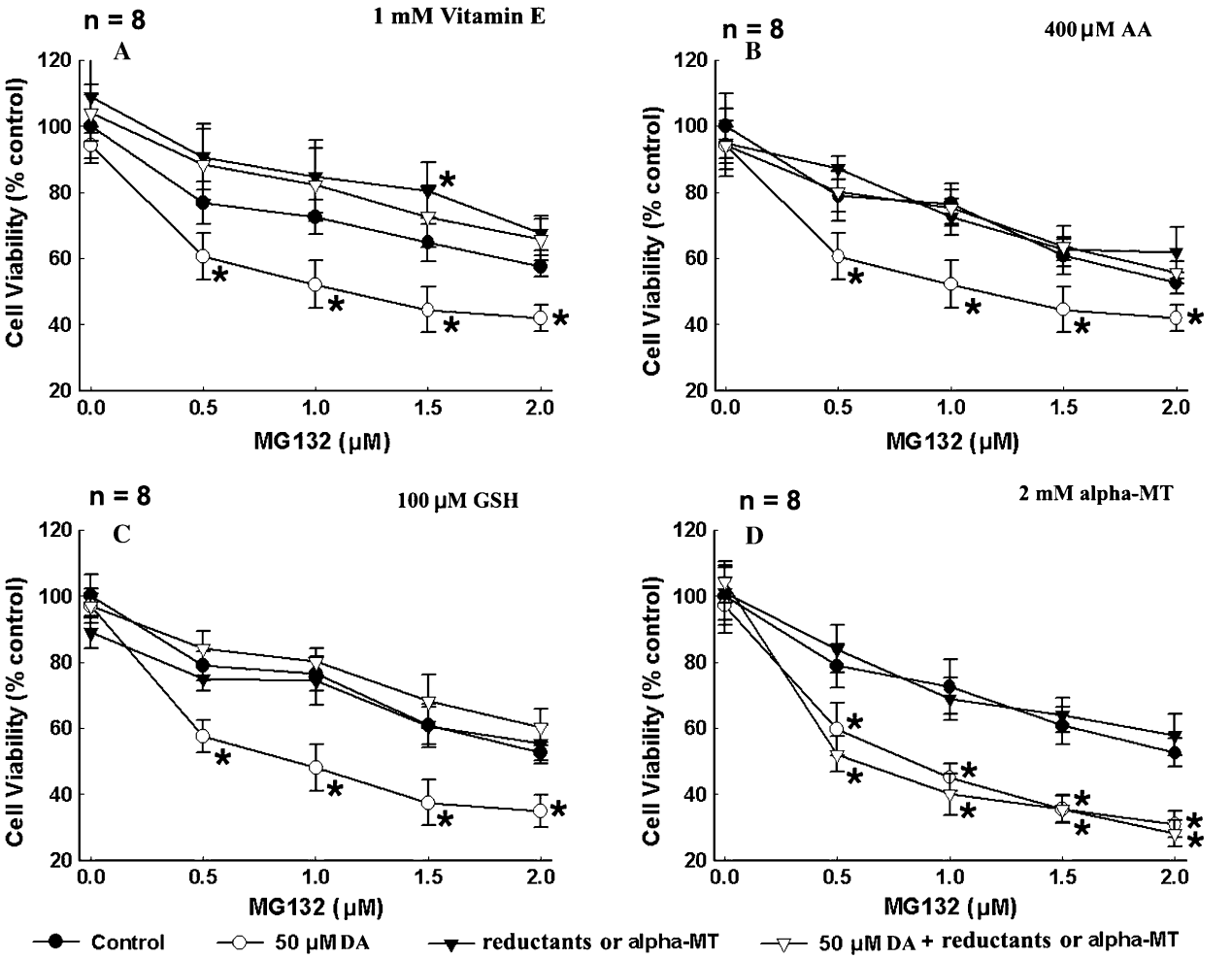

Figure 3. Exogenous DA could aggravate MG132-induced cell viability decrease in non-dopaminergic N2A cells, and its effect could be abrogated by reductants but could not by alpha-MT. N2A cells were challenged with MG132 overnight with or without exogenous DA, in the presence of Vitamin E (A), AA (B), GSH (C) and alpha-MT (D). Cells without challenge acted as controls. Viability of cells were detected by MTT assay and shown as '% control'. * at least $p < 0.05$, compared with cells challenged with same concentration of MG132 only.

N2A cells could take in exogenous DA into cytosol

Our HPLC study further disclosed that N2A cells do not contain DA endogenously but they could actually absorb a considerable amount of exogenous DA into their cytosol in an incubation time (1–3 h) and DA concentration dependent manner although they took in less DA compared with that by dopaminergic PC12 cells (Figure 4B and C). The DA content in PC12 and N2A cells could increase with time when cells were treated in DMEM with 800 μm DA for 1–3 h (Figure 4B). N2A cells and PC12 cells could take in a significant amount of DA when cells were treated with 50 μm DA alone for 3 h (Figure 4C).

Culture of dopaminergic cells with alpha-MT and reductants could improve proteasome activity of dopaminergic cells

We demonstrated that culture of dopaminergic MN9D cells in the presence of 2 mm alpha-MT or culture with different concentration of reductants overnight could significantly ameliorate the proteasome activity of dopaminergic MN9D cells and alleviate the effect of proteasome inhibition by MG132 (Figure 5A and B). However, culture of N2A cells with alpha-MT and various reductants could not influence the proteasome activity of N2A cells (data not shown). This indicated the inhibition of proteasome activity by endogenous DA in dopaminergic cells.

Culture of non-dopaminergic cells with exogenous DA could decrease proteasome activity of non-dopaminergic cells, which could be abrogated by reductants

Furthermore, challenge of N2A cells with exogenous DA overnight could decrease the proteasome activity and sensitize proteasome activity of N2A cells to MG132 inhibition in a DA dosage dependent manner (from 50–400 μm) (Figure 5C). The decrease of proteasome activity and sensitization of proteasome

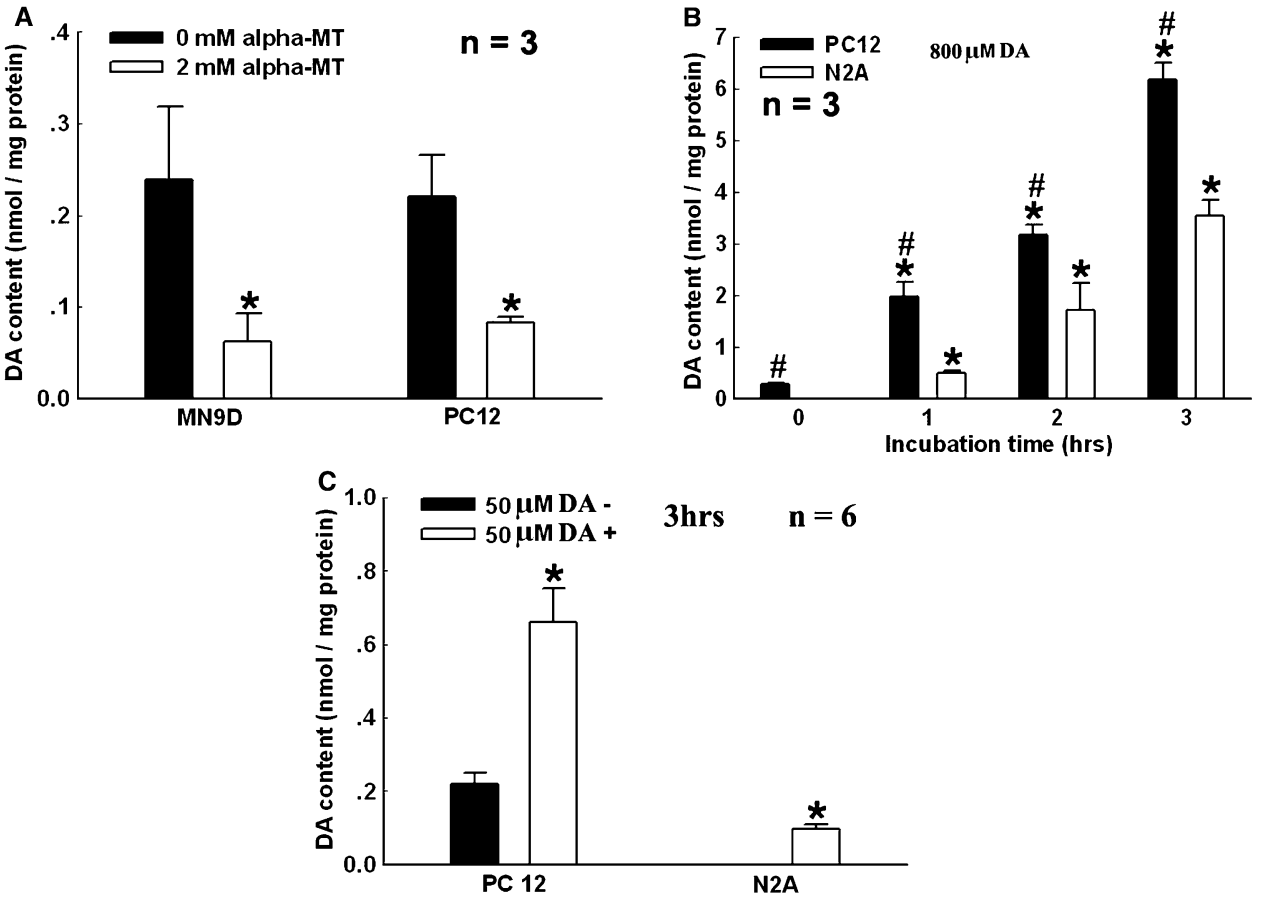

Figure 4. HPLC detection of DA content in MN9D, PC12 and N2A cells. (A), alpha-MT could significantly decrease DA content in dopaminergic MN9D and PC 12 cells. Cells were maintained in 2 mm alpha-MT containing DMEM for one passage before they were harvested for HPLC detection of DA content in cells. *at least $p < 0.05$, compared with cells without alpha-MT treatment. (B) and (C), PC12 and N2A cells could take in exogenous DA into cytosol to different extents. Cells were incubated in DMEM with 800 μM DA for 0–3 h (B) or with 50 μM DA for 3 h (C) before they were harvested for HPLC detection of DA content in cells. *at least $p < 0.05$, compared with cells without DA challenge; # at least $p < 0.05$, compared with N2A cells challenged for same period of time.

activity to MG132 inhibition due to DA challenge of N2A cells could be abrogated by various reductants (Figure 5D). Therefore, we concluded that endogenous DA in dopaminergic cells could function to inhibit proteasome activity and even co-operate with proteasome inhibitor to further decrease proteasome activity in dopaminergic cells.

Alpha-MT could decrease MDA content in dopaminergic cells

We further demonstrated that culture of dopaminergic MN9D and PC12 cells in the presence of 2 mm alpha-MT could significantly decrease their MDA content (Figure 6A). However, such a phenomenon could not be found when non-dopaminergic N2A cells had been similarly cultured in the presence of 2 mm alpha-MT (Figure 6A). The reduction in MDA content indicated the decreased lipid peroxidation level in dopaminergic cells when the level of endogenous DA decreased. Therefore the endogenous DA in dopaminergic cells could significantly lead to increased oxidative stress and even oxidative damage to dopaminergic cells.

Culture of non-dopaminergic cells with exogenous DA could increase MDA content, which could be abrogated by reductants

Challenge of N2A cells with exogenous DA (50–400 μm) overnight could increase MDA content in N2A cells in a DA dosage dependent manner (Figure 6B). However, the increase of MDA content in N2A cells due to challenge of 50 μm DA could be abrogated by various reductants (Figure 6C). The MDA content of N2A cells challenged with 50 μm DA overnight in the presence of 100 μm GSH or 400 μm AA together with 1 mm Vitamin E were even lower than that of control cells (Figure 6C).

Culture of non-dopaminergic cells with exogenous DA could increase the level of cellular ubiqitinated proteins

Western blot analysis using anti-ubiquitin antibodies demonstrated that challenge of N2A cells with

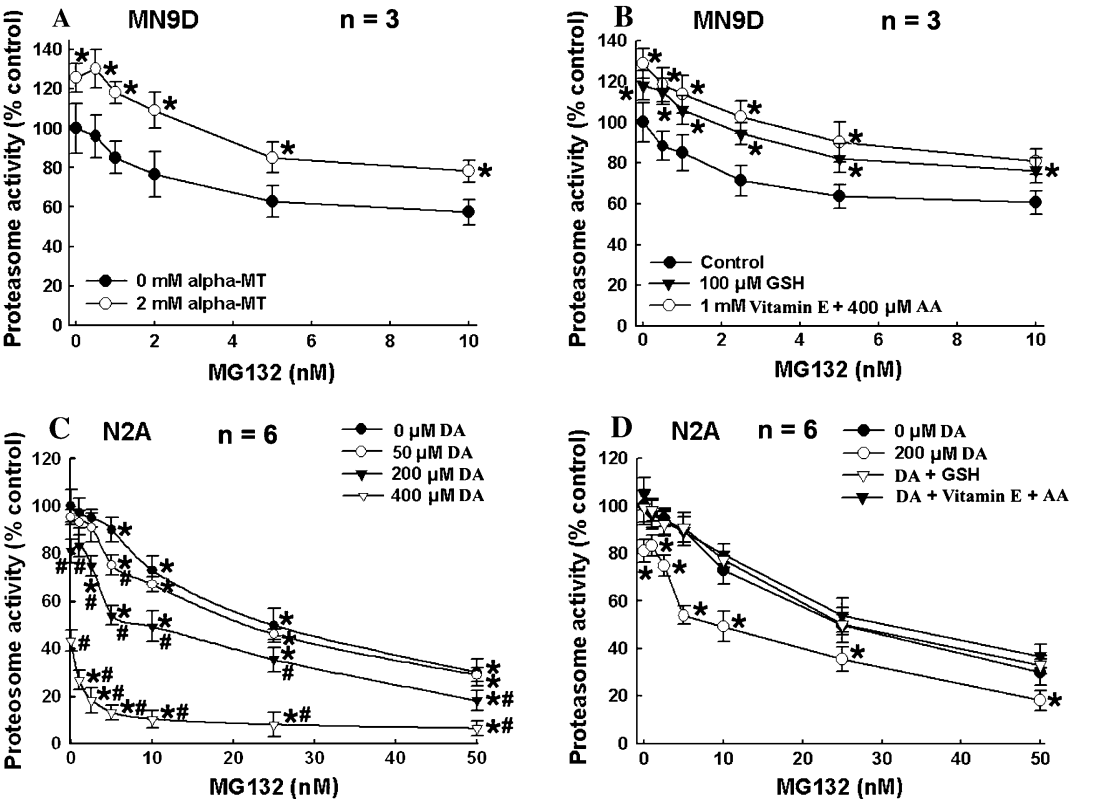

Figure 5. DA-related proteasome inhibition and co-operation with MG132 could be alleviated by reductants or alpha-MT. After cells were challenged with or without DA, alpha-MT or reductants, cells were harvested and cell extracts were further incubated with different concentration of MG132 for 30 min. Then fluorogenic substrate was added and further incubated for 30 min before detection of proteasome activity. (A and B) MN9D cells were cultured (A) in the presence of 2 mm alpha-MT or (B) with reductants overnight, *at least $p < 0.05$, compared with proteasome activities of cells without alpha-MT or reductants but with same concentration of MG132 challenge. (C) N2A cells were cultured with 0, 50, 200 or 400 μM DA overnight, *at least $p < 0.05$, compared with proteasome activity of cells without MG132 but with same concentration of DA challenge; # at least $p < 0.05$, compared with proteasome activity of cells without DA challenge but with same concentration of MG132. (D) N2A cells were cultured with 0 μM DA, 200 μM DA, 200 μM DA plus 100 μM GSH or 200 μM DA plus 1 mM Vitamin E and 400 μM AA overnight. *at least $p < 0.05$, compared with proteasome activity of cells with 0 μM DA but with same concentration of MG132.

exogenous DA (50–400 μm) overnight could significantly increase the level of ubiqitinated proteins in the cytosol of N2A cells (Figure 6D). The level of ubiqitinated proteins began to increase even when N2A cells were challenged only with 50 μm DA overnight and the level further increased significantly in a DA concentration dependent manner (Figure 6D).

Culture of non-dopaminergic cells with exogenous DA could induce inclusion body formation

Immunochemistry staining disclosed that challenging N2A cells with 400 μm exogenous DA overnight could induce formation of anti-ubiquitin antibody recognized inclusion bodies in the cytosol (Figure 6F). Such inclusion bodies could not be found in N2A cells without DA challenge (Figure 6E). The inclusion bodies were absent in N2A cells challenged with 50–200 μm exogenous DA overnight (data not shown).

Discussion

In this study we confirmed that the endogenous DA in two dopaminergic cell lines (MN9D and PC12) was the factor making them vulnerable to challenge by proteasome inhibitor MG132. We also found that viability of these cells was highly sensitive to MG132 challenge which could be alleviated by various reductants as well as by specific TH inhibitor, alpha-MT. However reductants and alpha-MT failed to alleviate the non-dopaminergic N2A cell viability decrease caused by MG132 challenge. The alpha-MT is not a reductant but could inhibit TH and could significantly decrease the endogenous DA level in dopaminergic MN9D and PC12 cells in current study as well as in our previously published study [19]. The ability of endogenous DA to sensitize cell viability to MG132 challenge was further supported by our experiments applying exogenous DA to non-dopaminergic N2A cells. N2A cells could take in considerable amounts of exogenous DA into the cytosol, even when treated

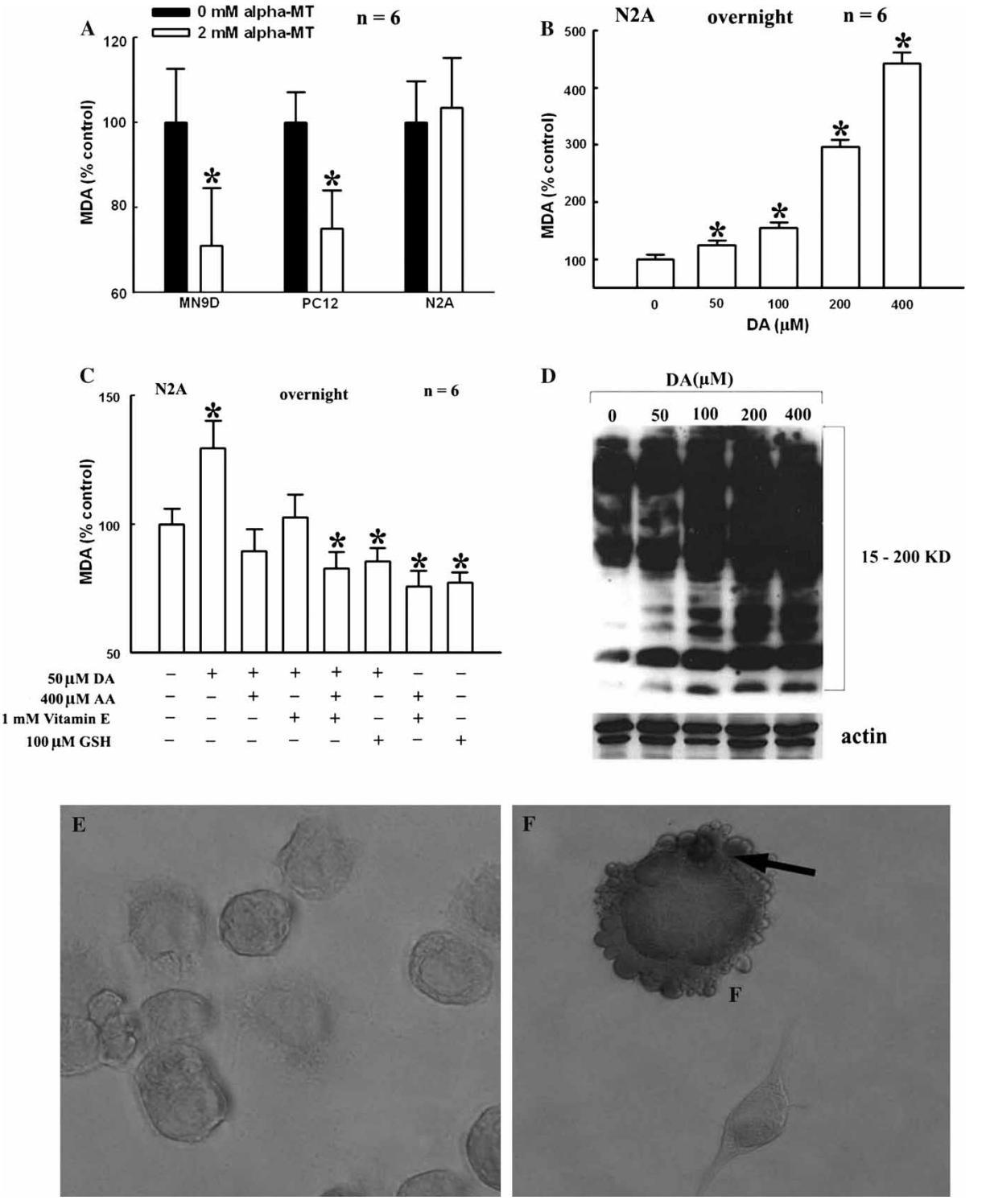

Figure 6. DA induced increase of lipid peroxidation in MN9D, PC12 and N2A cells as well as protein ubiquitination and inclusion body formation in N2A cells. (A) MN9D, PC12 and N2A cells were cultured in the presence of 2 mm alpha-MT. Cells were harvested and MDA content was detected and shown as '% control'. *at least $p < 0.05$, compared with MDA content of cells without alpha-MT treatment. (B) exogenous DA could increase MDA content of N2A cells. N2A cells were challenged with 0–400 μM DA overnight. Cells were harvested and MDA content was detected. Cells without DA challenge acted as controls. *at least $p < 0.05$, compared with MDA content of control cells. (C) exogenous DA induced increase of MDA content could be abrogated by reductants. N2A cells were challenged with or without 50 μM DA in the presence or absence of different concentration of AA, Vitamin E or GSH overnight. Cells without any challenge acted as controls. *at least $p < 0.05$, compared with MDA content of control cells. (D) exogenous DA induced increase of ubiquitination of proteins in N2A cells. N2A cells were challenged with 0–400 μM DA overnight. Cells were harvested, lysed and analysed by western blot using mouse anti-ubiqutin antibody. (E) and (F) DA induced inclusion body formation in N2A cells. N2A cells were challenged with 0 (E) or 400 (F) μM DA overnight. Cells were stained with immunochemistry using mouse anti-ubiqutin antibody and observed under microscopy with 400 times amplifications. The inclusion body (black arrow) was found in N2A cell challenged with 400 μM DA.

with only a sub-lethal dosage of exogenous DA. The sub-lethal dosage of exogenous DA could increase the sensitivity of N2A cell viability to MG132 challenge, which could be alleviated by reductants but not by alpha-MT. Furthermore, challenging N2A cells with sub-lethal dosage of DA was enough to increase the level of lipid peroxidation and ubiquitinated proteins in them as well as to sensitize their proteasomes to proteasome inhibitor MG132 challenge. Challenging N2A cells with higher DA concentration could even lead to inclusion bodies formation in their cytosol. Taken together, these data demonstrated that endogenous DA in dopaminergic cells could stress dopaminergic cells significantly via increase of oxidative stress, damnification of functional proteins and impairment of UPS functions. The endogenous DA in dopaminergic cells could contribute to the vulnerability and even degeneration of dopaminergic cells. Our unpublished data demonstrated that reductants, such as GSH, AA and Vitamin E, could directly inhibit DA oxidation in solutions, especially by GSH. Therefore inhibition of endogenous DA oxidation by reductants or decrease of DA level by TH inhibitor in dopaminergic cells could potentially protect dopaminergic cells against proteasome malfunction due to endogenous or exogenous factors.

It is well established that DA oxidation could produce ROS and toxic DA quinones [6]. The modification or conjugation of protein by DA oxidative metabolites could lead to functional protein modification and inactivation [20]. The UPS is the primary proteolytic complex responsible for the elimination of unwanted and misfolded intracellular proteins [3]. We hypothesize that the endogenous DA might aggravate the cell viability decrease in the presence of malfunction of UPS at least via two mechanisms. The first mechanism is that DA-modified proteins in dopaminerigc neurons need to be degraded and eliminated by UPS in time. The malfunction of UPS will cause accumulation and aggregation of these DA-modified proteins in cytosol, leading to impairment of dopaminergic neurons. This was supported by findings that DA could enhance the formation of aggregates under proteasome inhibition in PC12 cells [9]. The second mechanism is that endogenous DA could directly modify and inactivate components of UPS and impair UPS functions, as demonstrated by previous reports [7,21] and also findings in this study. Thus, the impairment of UPS could become more serious in dopaminergic neurons than in non-dopaminergic cells. Therefore we conjecture that the endogenous DA in dopaminergic neurons could significantly promote selective dopaminergic neurodegeneration under the conditions of exopathic or idiopathic UPS defects, which could be potentially alleviated by reductant remedy. Our experiments could be extended to animal models to ascertain if treatment to inhibit endogenous DA oxidation by reductants or decrease of DA level by TH inhibitor may help to control PD onset and development.

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