Free Radical Research, April 2009; 43(4): 417–430

informa
healthcare

Dopamine (DA) induced irreversible proteasome inhibition via DA derived quinones

ZHI DONG ZHOU & TIT MENG LIM

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

(Received 20 December 2008; revised 3 February 2009)

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

Abstract
This study demonstrated that DA and its oxidative metabolites: H_2O_2 and aminochrome (AM), cyclized DA quinones, could all directly inhibit proteasome activity. DA and AM, especially AM, could induce intensive and irreversible proteasome inhibition, whereas proteasome inhibition induced by H_2O_2 was weaker and GSH reversible. It was concluded that DA induced irreversible proteasome inhibition via DA-derived quinones, rather than through small molecular weight ROS. The AM was also more toxic than H_2O_2 to dopaminergic MN9D cells. Furthermore the cytotoxicity and proteasome inhibition induced by DA, AM and H_2O_2 could be abrogated by GSH, ascorbic acid (AA), Vitamin E, SOD (superoxidase dismutase) or CAT (catalase) with different profiles. Only GSH was potent to abrogate DA, AM or H_2O_2-induced cell toxicity and proteasome inhibition, as well as to reverse H_2O_2-induced proteosome inhibition. Therefore, therapeutic strategies to increase GSH level or to use GSH substitutes should function to control PD onset and development.

Keywords: *Aminochrome, dopamine, GSH, H_2O_2, Parkinson's disease, proteasome inhibition*

Introduction

It has been hypothesized that dopamine (DA)-related oxidative stress might be a key factor related to dopaminergic neurodegeneration of Parkinson's disease (PD) [1]. However, DA is known to undergo a complex series of metabolic events. Besides very small reactive oxygen species (ROS) such as H_2O_2, oxidation of DA could simultaneously generate highly reactive DA *o*-quinone, which could cyclize automatically to form cyclized DA quinone, known as aminochrome (AM) at physiological pH. The AM could rearrange to form 5,6-dihydroindole and finally polymerize to form melanin [2,3]. DA quinones are highly reactive and could covalently react with the cysteine residues of comprehensive functional proteins and irreversibly inhibit their activity [4,5]. Recent studies suggested the highly reactive DA-derived DA quinones rather than ROS play an important role in DA-induced toxicity [6–8]. On the other hand, malfunction of the ubiquitin-proteasome system (UPS), a complicated protein complex responsible for clearing unwanted and misfolded proteins, has been implicated to be closely related to dopaminergic neurodegeneration and protein aggregates found in PD [9]. Inhibition or failure of UPS could lead to accumulation and aggregation of proteins and dopaminergic cell demise [10]. It has been proposed that defects in UPS are a universal point underlying both the familial and sporadic forms of PD [11]. Different mutants in the gene encoding parkin, an ubiqutin ligase, have been detected in autosomal recessive juvenile PD patients [12]. In addition, a mutation in the gene encoding ubiquitin C-terminal hydrolase L1 (UCH-L1) has been reported in rare cases of familial PD [13]. Defects in protein handling were also reported in sporadic PD where the levels of oxidized proteins were increased and Lewy body could be found in degenerating neurons [14].

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

ISSN 1071-5762 print/ISSN 1029-2470 online © 2009 Informa UK Ltd.
DOI: 10.1080/10715760902801533

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

To date, complicated interactions between DA and UPS related to dopaminergic neurodegeneration of PD has been demonstrated [15–19]. Recently, DA quinones derived from DA were found to be implicated to proteasome activity inhibition [15]. DA was also found to aggravate cytotoxicity caused by proteasome inhibitor [16]. Furthermore, DA was found to enhance the formation of aggregates under proteasome inhibition in PC12 cells [17]. Inhibition of proteasome activity was found to sensitize DA neurons to protein alterations and oxidative stress [20]. Researchers also demonstrated that application of the proteasome inhibitor could increase the toxicity of DA and the levels of protein oxidation following administration of DA [18]. Our recent findings demonstrated that endogenous DA could render dopaminergic cells vulnerable to challenge by proteasome inhibitor MG132 [19]. These studies implicated the synergistic effects on UPS malfunction and dopaminergic neurodegeneration induced by DA and UPS inhibitors.

However, the detailed mechanism for DA-induced proteasome inhibition is still illusive. In this study, we demonstrated for the first time that DA could induce intensive and irreversible but preventable proteasome inhibition via its oxidative metabolites cyclized DA quinones, rather than through its oxidative metabolite H_2O_2. Based on these findings, DA and its oxidative metabolites could be considered as endogenous UPS inhibitors, which could contribute to dopaminergic cells vulnerability and even selective dopaminergic neurodegeneration of PD. We also established in this study that GSH was an important factor to protect dopaminergic cells against DA and its oxidative metabolites induced toxicity via abrogation of DA oxidation; scavenge of ROS as well as detoxification of highly reactive DA derived quinones.

Materials and methods

Chemicals

Ascorbic acid (AA), bovine serum albumin (BSA), dopamine (DA), Dulbecco's modified Eagle's Medium (DMEM) powder, proteasome inhibitor MG132 (dissolved in dimethyl sulphoxide), EDTA, glutathione (GSH), HEPES, sodium dodecyl sulphate (SDS), tyrosinase (from mushroom), catalase (CAT), superoxidase dismutase (SOD) and Vitamin E were all purchased from Sigma (USA).

AM preparation

AM was prepared through rapid catalysis of DA by tyrosinase. Usually, 1 mM AM was produced by reaction of 1 mM DA (dissolved in distilled water) with 100 units of tyrosinase (1 units/μl, 2000 units/mg, Mw ~125 000, freshly prepared in distilled water) in 1 ml distilled water for ~10 min at room temperature with constant shaking. Different concentrations of AM were then diluted and used for experiments immediately.

Cell lines and toxicity studies

Dopaminergic neuronal MN9D [21] cells were cultured and maintained in DMEM with 10% (v/v) foetal bovine serum (FBS) (Clonetech, USA) and 1% (v/v) penicillin-streptomycin (Sigma, USA). For toxicity study of DA, H_2O_2 and MG132, cells cultured in DMEM were exposed to different concentration of DA, H_2O_2 or MG132 in the presence or absence of different concentration of GSH, AA or Vitamin E for different time. Cells were then subjected to MTT analysis after 24 h or harvested for cell extracts after indicated time. For toxicity study of AM and toxicity comparison with that of H_2O_2, cells were first cultured in DMEM medium. The DMEM medium was changed to phosphate buffered saline (PBS) just before administering AM or H_2O_2. Then different concentrations of H_2O_2 or freshly prepared AM were added together with or without different concentrations of GSH, AA, Vitamin E, SOD, AA or BSA. The H_2O_2 or AM was replenished every hour. Cells were cultured under challenge in PBS for 3 h. After that, cells were harvested for trypan blue staining or cell extracts preparation.

MTT assay

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).

Trypan blue exclusion analysis

Cells were harvested by trypsinization. The cell suspension was mixed with same volume of 2% (w/v) trypan blue solution and cells were counted under a light microscope (Axiovert 25 Zeiss, Germany). The numbers of total cells and dead cells were counted and the percentage of dead cells was determined. At least 200 cells were counted for each cell suspension sample.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

Preparation of cell extract for proteasome activity detection

Proteasome chymotrypsin peptidase activity was determined by procedures as previously reported [22,23]. 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 [19,24]

Briefly, cell extracts were prepared and protein content was quantified. For each sample, 5 μl cellular extract (containing 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 DA, AM, H_2O_2 and/or MG-132 as well as GSH, AA or Vitamin E were added to the cellular extract and incubated at 37°C for different times. After that 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, for chymotrypsin-like activity; Benzyloxycarbonyl-leu-leu-glu-7-amido-4-methylcoumarin, Biomol, USA, for caspase-like activity; Benzoyl-val-gly-arg-7-amido-4-methylcoumarin, Biomol, USA, for trypsin-like activity)) 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. We mainly focused on the inhibition of chymotrypsin-like activity of proteasome by DA, AM and H_2O_2, therefore all indicated proteasome activities without specific illumination should be the chymotrypsin-like activity of proteasomes, unless specifically clarified.

Dialysis experiments

MN9D cell extracts were incubated with or without DA, AM or H_2O_2 in buffer A at 37°C for 3 h and then dialysed against buffer A overnight at 4°C in a dialysis tube (Float-A-Lyzer, MWCO 100 KD, Spectrum Laboratories Inc, USA) with at least two changes of dialysis buffer A. Then the dialysed samples were subjected to further spectral analysis or proteasome activity detection.

Incubation of DA or AM in solutions

DA or AM freshly prepared was incubated in distilled water or buffer A in the presence or absence of different concentration of SOD, CAT or BSA for different times. Samples were then analysed by HPLC or spectrometric scanning.

Detection of DA oxidation by HPLC

The oxidation of DA after incubation in solutions in the presence or absence of SOD, CAT or BSA in buffer A was detected by a HPLC procedure developed by ourselves [25]. We used two elution buffer systems (elution buffer A and elution buffer B). The elution buffer A was 8% methanol (v/v), 0.1 M sodium phosphate, 20 mM sodium 1-heptane-sulphonate, 0.1 mM EDTA, adjusted to pH 4.75 with NaOH. The elution buffer B was 80% (v/v) acetonitril in distilled water. The HPLC procedure was set up and performed as follows: the flow rate was 1 ml/min; the column was first equilibrated with 1 CV elution buffer A. 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 of elution buffer B (volume of elution buffer B/volume of column wash (%)) began. The start concentration of elution buffer B (80% (v/v) acetonitril in distilled water) 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 the gradient delay of 5 CV.

Spectral analysis

Samples containing DA or AM in PBS or buffer A with or without 25 mg/ml BSA underwent spectral analysis by measuring the separate absorbance (scanning from 340–800 nm wavelength, step: 5 nm wavelength) using spectrophotometer SpectraMAX 250 with software SOFTmax PRO 4.3 LS.

Data analysis

An HPLC chromatogram was analysed and exported from Software UNICON 4.11. 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

DA, AM and H_2O_2 could directly inhibit proteasome activity of dopaminergic MN9D cells to different extent

Incubation of cell extracts of dopaminergic MN9D cells in buffer A with 100–800 μM DA, 12.5–100 μM AM or 50–2000 μM H_2O_2, respectively, for 1–3 h at 37°C could lead to different extents of proteasome inhibition in an incubation time and/or toxins dosage dependent manner (Figure 1). The chymotrypsin-like activity, trypsin-like activity and caspase-like activity of proteasome were tested under challenges by DA,

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

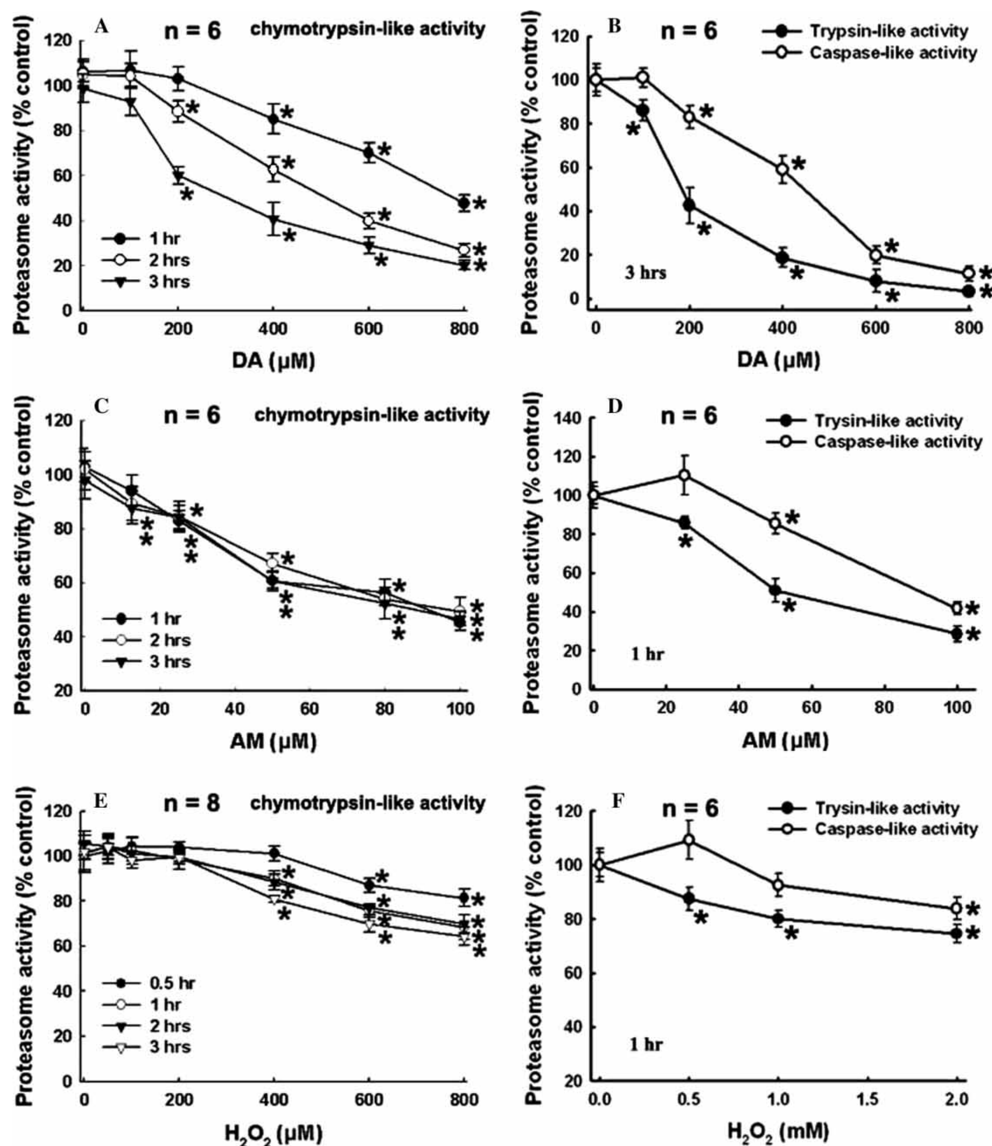


Figure 1. Proteasome inhibition induced by DA, AM and H_2O_2. Cell extracts of dopaminergic MN9D cells were challenged, respectively, with DA (A and B), AM (C and D) or H_2O_2 (E and F) for 1–3 h at 37°C. Then different proteasome substrates was added subsequently and further incubated for 30 min before determination of chymotrypsin-like activity, trypsin-like activity and caspase-like proteasome activities by fluorescence measurement. Proteasome activity of cell extracts was shown as '% control'. Proteasome activities without any challenge were as 'controls' (* at least $p < 0.05$, compared with controls).

AM and H_2O_2. We found that these different kinds of proteasome activities could all be inhibited by DA, AM and H_2O_2 to different extents (Figure 1). However the caspase activity of proteasome was a little resistant to toxins challenges compared with the inhibition effect of the other two proteasome activities induced by DA, AM and H_2O_2 (Figure 1). Of the three toxic factors, the proteasome inhibition induced by AM was the most distinct. Significant chymotrypsin-like activity of proteasome activity decrease could be achieved when cell extracts had been challenged with 12.5 μM AM for 1 h while no significant proteasome activity decrease could be detected when challenged with 200 μM H_2O_2 or 200 μM DA for the same duration (Figure 1A, C and E). The proteasome inhibition induced by H_2O_2 was weaker than those by DA and AM. About 50% decrease of chymotrypsin-like control proteasome activity could be achieved by challenge of cell extracts for 1 h with 800 μM DA or 100 μM AM while challenge with 800 μM H_2O_2 for the same duration could only induce $\sim 20\%$ decrease of control proteasome activity (Figure 1A, C and E). It was noted that the inhibition of chymotrypsin-like proteasome activity by DA could significantly exacerbate with time (Figure 1A). While inhibitions of chymotrypsin-like proteasome activity by AM and H_2O_2 did not significantly exacerbate from 1 h to 3 h, especially

RIGHTSLINK

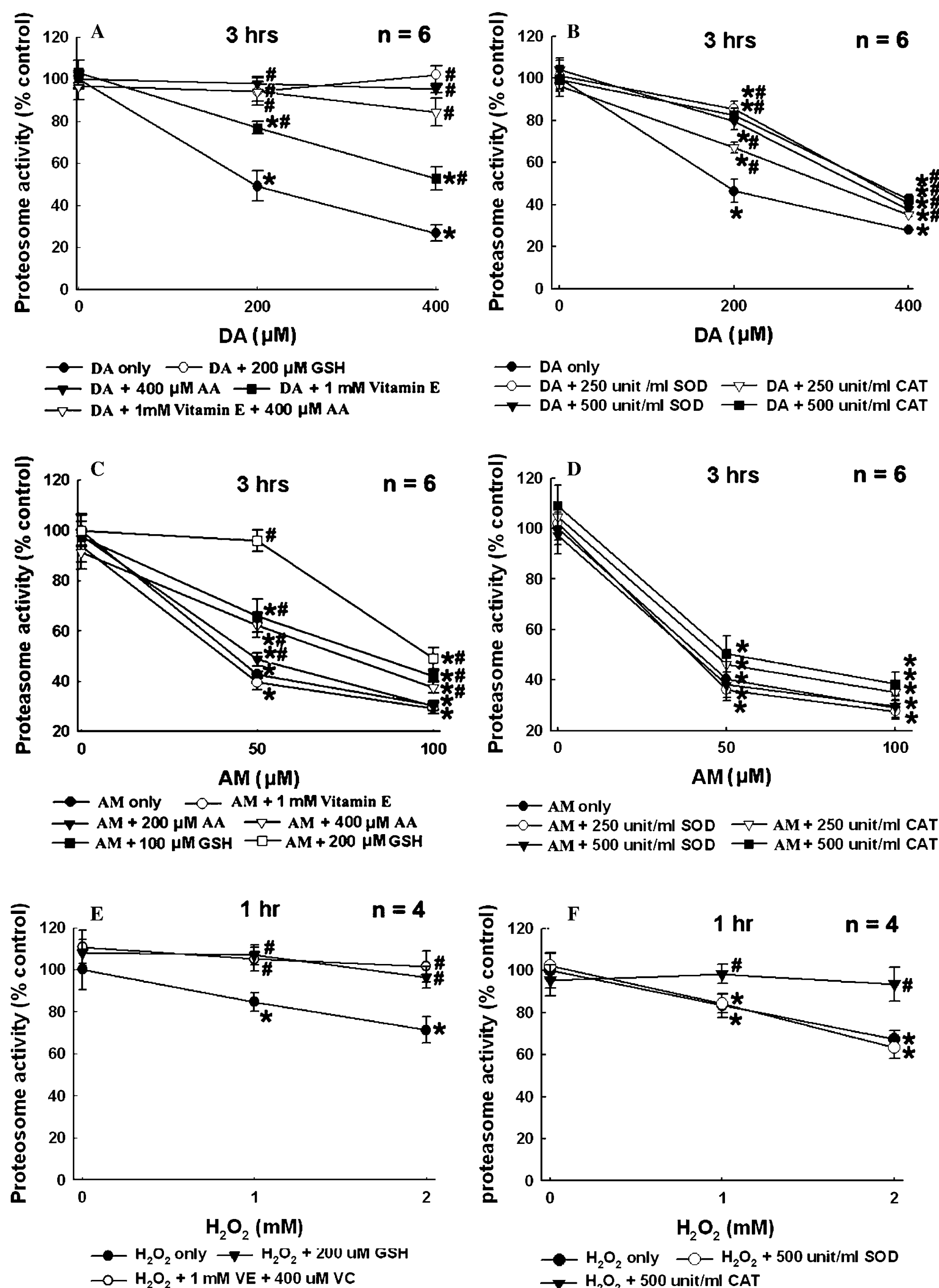


Figure 2. Alleviation of inhibition of chymotrypsin-like proteasome activity induced by DA, AM and H_2O_2 with GSH, AA, Vitamin E, SOD and CAT. Cell extracts of dopaminergic MN9D cells were challenged with DA (A and B), AM (C and D) or H_2O_2 (E and F) in the presence or absence of GSH, SOD, CAT Vitamin E and/or AA for 1 or 3 h at 37°C. Then proteasome substrate was added subsequently and further incubated for 30 min before determination of proteasome activity by fluorescence measurement. Proteasome activity of cell extracts was shown as '% control'. Proteasome activities without any challenge were as 'controls' (* at least $p < 0.05$, compared with controls; # at least $p < 0.05$, compared with proteasome activities challenged with same concentration of DA, AM or H_2O_2 but without GSH, SOD, CAT, Vitamin E or AA).

by AM (Figure 1C and E). On the other hand, the dosage effect of proteasome inhibition by H_2O_2 was not as distinct as those by DA and AM (Figures 1 and 2E and F). Challenge of cell extracts with 1 and 2 mM H_2O_2 could not achieve a more dramatic decrease of chymotrypsin-like proteasome activity compared with those challenged by 800 μM H_2O_2 (Figure 1E and 2E and F).

The inhibition of chymotrypsin-like proteasome activity induced by DA, AM and H_2O_2 could be abrogated by GSH, Vitamin E, AA, SOD and CAT with different profiles

Our following extensive studies then focused on the inhibition effect of chymotrypsin-like proteasome activity. Co-incubation of cell extracts with 200 μM

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

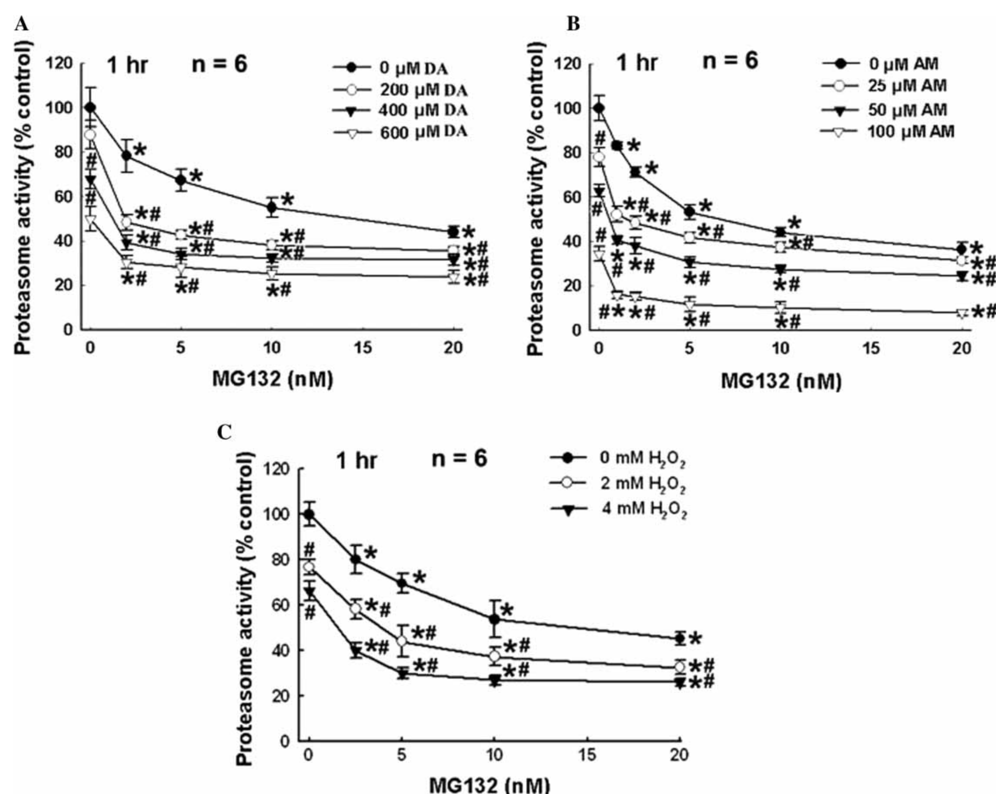


Figure 3. Cooperation of DA, AM and H_2O_2 with proteasome inhibitor MG132 leading to aggravated inhibition of chymotrypsin-like proteasome activity. Cell extracts of MN9D cells were challenged with DA (A), AM (B) or H_2O_2 (C) in the presence of 0–20 nM MG132 at 37°C for 1 h. Then proteasome substrate was added and further incubated for 30 min before determination of proteasome activity by fluorescence measurement. Proteasome activities of cell extracts without any challenge were as 'controls'. Proteasome activity was shown as '% control' (* at least $p < 0.05$, compared with proteasome activity of cell extracts challenged with same concentration of DA, AM or H_2O_2 but without MG132; # at least $p < 0.05$, compared with proteasome activity of cell extracts challenged with same concentration of MG132 but without DA, AM or H_2O_2).

GSH, 400 μM AA or 1 mM Vitamin E plus 400 μM AA, respectively, together with 200–600 μM DA could completely abrogate the DA-induced proteasome inhibition, whereas 1 mM Vitamin E alone could only partially prevent the DA-induced proteasome inhibition (Figure 2A). However, SOD and CAT could partially prevent DA-induced proteasome inhibition (Figure 2B). The preventive effect against AM-induced proteasome inhibition by reductants was poorer, compared with the effect of reductants to abrogate DA-induced proteasome inhibition (Figure 2A and C). GSH and AA could protect against AM-induced proteasome inhibition in a GSH and AA concentration dependent manner while Vitamin E did not have any protective effect against AM-induced proteasome inhibition (Figure 2C). GSH at 200 μM could completely abrogate the proteasome inhibition induced by 50 μM AM challenge for 3 h (Figure 2C). However, 200 μM GSH could only partially prevent the proteasome inhibition induced by 100 μM AM challenge for 3 h (Figure 2C). The protective effect of AA against AM-induced proteasome inhibition was inferior to that of GSH. AA at 400 μM could only partially abrogate the proteasome inhibition induced by 100 μM AM challenge for 3 h, while 200 μM AA could not significantly abrogate 100 μM AM-induced proteasome inhibition for 3 h (Figure 2C). On the other hand, SOD and CAT did not have a significant effect in protection against AM-induced proteasome inhibition (Figure 2D). We also demonstrated that reductants were quite competent to protect against H_2O_2-induced proteasome inhibition, even when a very high concentration of H_2O_2 was applied (Figure 2E). GSH at 200 μM or 1 mM Vitamin E plus 400 μM AA could completely abrogate proteasome inhibition induced by challenge with 1 or 2 mM H_2O_2 for 1 h (Figure 2E). However, 500 unit/ml CAT could completely abrogate 1 or 2 mM H_2O_2-induced proteasome inhibition while SOD had no effect on H_2O_2-induced proteasome inhibition (Figure 2F).

DA, AM and H_2O_2 could cooperate with proteasome inhibitor MG132 leading to aggravated inhibition of chymotrypsin-like proteasome activity

Challenge of cell extracts with 0–20 nM MG132 could induce MG132 dosage dependent proteasome activity decrease (Figure 3). However, DA, AM and

RIGHTSLINK

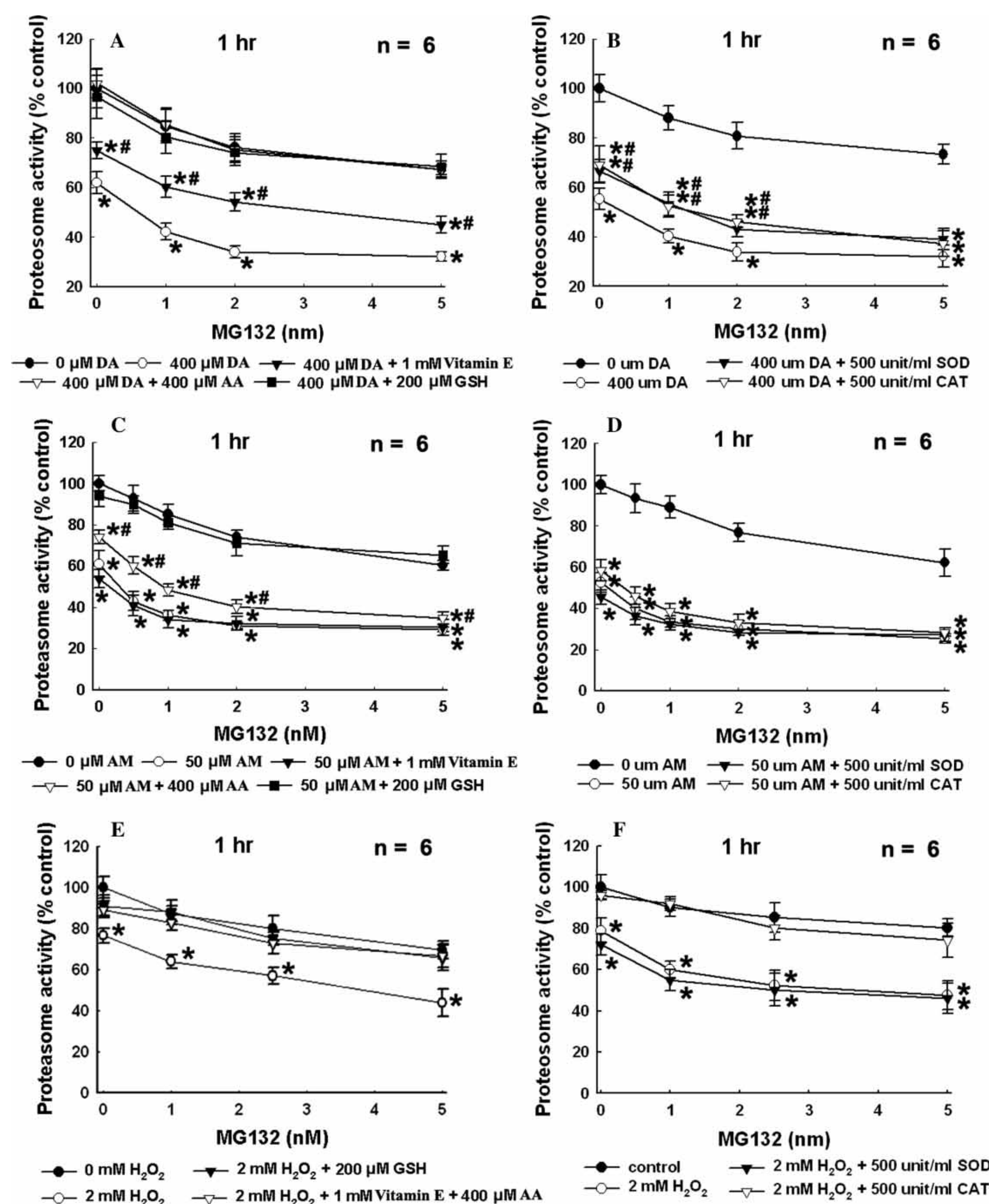


Figure 4. The aggravated inhibition of chymotrypsin-like proteasome activity induced by cooperation of proteasome inhibitor MG132 with DA, AM or H_2O_2 could be abrogated by GSH, SOD, CAT, Vitamin E and AA. Cell extracts of MN9D cells were challenged with 600 μM DA (A and B), 50 μM AM (C and D) or 2 mM H_2O_2 (E and F) together with 0–5 nM MG132 in the presence or absence of GSH, SOD, CAT, Vitamin E and/or AA for at 37°C for 1 h. Then proteasome substrate was added and further incubated for 30 min before determination of proteasome activity by fluorescence measurement. Proteasome activities of cell extracts without any challenge were as 'controls'. Proteasome activity was shown as '% control' (* at least $p < 0.05$, compared with proteasome activity of cell extracts challenged with same concentration of MG132 but without DA, AM or H_2O_2; # at least $p < 0.05$, compared with proteasome activity of cell extracts challenged with same concentration of MG132 with 600 μM DA or 50 μM AM but without GSH, SOD, CAT, Vitamin E and AA).

H_2O_2 were found to be able to cooperate with MG132 and lead to aggravated proteasome inhibition (Figure 3). Compared with the proteasome activity of cell extracts challenged with MG132 only, challenge of cell extracts with 200–600 μM DA, 25–100 μM AM or 2–4 mM H_2O_2, respectively, plus 1–20 nM MG132 for 1 h would lead to aggravated proteasome inhibition, especially when challenged with low concentration (1–2.5 nM) of MG132 (Figure 3). The synergistic effect of proteasome inhibition dependent on the concentrations of DA, AM, or H_2O_2 as well as MG132 applied (Figure 3).

The synergistic inhibition of chymotrypsin-like proteasome activity induced by MG132 plus DA, AM or H_2O_2 could be abrogated by GSH, Vitamin E, AA, SOD and CAT

We demonstrated that the cooperation between 0–5 nM MG132 and 400 μM DA leading to aggravated proteasome inhibition could be completely abrogated by 200 μM GSH or 400 μM AA and incompletely abrogated by 1 mM Vitamin E (Figure 4A). Furthermore, the inhibition induced by MG132 plus 400 μM DA could be partially alleviated by 500 unit/ml SOD and CAT (Figure 4B); 200 μM GSH could also

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

RIGHTSLINK

completely abrogate the inhibition induced by 50 μM AM plus 0–5 nM MG132 (Figure 4C). However, the protective effect of 400 μM AA against synergistic proteasome inhibition induced by 50 μM AM plus 0–5 nM MG132 was partial, while 1 mM Vitamin E had no effect on the inhibition induced by 50 μM AM plus 0–5 nM MG132 (Figure 4C). We also demonstrated that SOD and CAT had no significant effects on the inhibition induced by AM plus MG132 (Figure 4D). On the other hand, the synergistic inhibition induced by 2 mM H_2O_2 plus MG132 could be completely abrogated by 200 μM GSH or 1 mM Vitamin E plus 400 μM AA (Figure 4E). The inhibition induced by H_2O_2 plus MG132 could also be completely abrogated by 500 unit/ml CAT but could not be influenced by 500 unit/ml SOD (Figure 4F).

The inhibition of chymotrypsin-like proteasome activity induced by DA and AM was irreversible while the proteasome inhibition by H_2O_2 was GSH reversible

We performed another set of experiments that involved a dialysis step. The dialysis was to allow the remaining DA, AM or H_2O_2 to leave the treated cell extracts, so that the proteasomes that had reacted with the DA and its metabolites could be subsequently tested for reversal of function without interference by the remaining DA and its metabolites. We first established that incubation of MN9D cell extracts with 200–600 μM DA, 25–50 μM AM or 400–800 μM H_2O_2 in buffer A for 3 h at 37°C (we called this treatment step Procedure A) could decrease proteasome activity (Figure 5). Next, cell extracts treated with Procedure A were dialysed in dialysis tubes overnight at 4°C (we called this dialysis step as Procedure B). We found that proteasome activities of cell extracts challenged with 100 or 200 μM DA would further decrease after going through Procedure B (Figure 5A and B). The aggravated proteasome inhibition after Procedure B could be explained by the extended time for any available DA to interact with the proteasome contained within the dialysis tube, since it would take time to completely separate the unbound DA through dialysis overnight. To finally test whether the proteasome inhibition could be reversed or not, we subjected the challenged cell extracts after procedure B to a further incubation with or without reductants, SOD or CAT for 3 h at 37°C (we called this incubation step Procedure C). We found that 200 μM GSH, 1 mM Vitamin E plus 400 μM AA, 500 unit/ml SOD or CAT could not reverse DA or AM-induced proteasome inhibition (Figure 5A–D). Interestingly, treatment of 400–800 μM H_2O_2 -challenged cell extracts with Procedure C in the absence of reductants, SOD or CAT would lead to further decrease of proteasome activity, compared with the proteasome activity with procedure B (Figure 5E and F). However the H_2O_2-induced proteasome inhibition could be completely reversed after incubation of H_2O_2-challenged cell extracts with 200 μM GSH in Procedure C while incubation of H_2O_2-challenged cell extracts with 1 mM Vitamin E plus 400 μM AA in Procedure C could only partially prevent the further decrease of proteasome activity (Figure 5E). The H_2O_2-induced proteasome inhibition could not be reversed after incubation of H_2O_2-challenged cell extracts with 500 unit/ml SOD and CAT (Figure 5F). Therefore, DA and AM induced intensive, preventable but irreversible proteasome inhibition; whereas H_2O_2-induced proteasome inhibition was weaker, preventable as well as GSH reversible.

DA, AM and H_2O_2 could be toxic to MN9D cells and could be alleviated by GSH, Vitamin E, AA, SOD, CAT as well as by 25 mg/ml BSA

We demonstrated that challenging MN9D cells with 100–200 μM DA overnight, 20–50 μM H_2O_2 overnight or 50–200 μM AM for 3 h could lead to significant MN9D cell death in a DA, AM or H_2O_2 dosage dependent manner (Figure 6). AM was found to be the most toxic to cells (Figure 6). About 80% of cells would die after they had been challenged with 200 μM AM only for 3 h (Figure 6C and D). Whereas challenge of MN9D cells for 3 h with 200 μM H_2O_2 could not induce significant cell death, suggesting that the toxicity of AM was much stronger than that of H_2O_2 (supplementary Figure). Remarkably, we also observed that the dead cells caused by AM challenge appeared as if they had been fixed onto the culture plates and were difficult to be detached by trypsination. Furthermore, we demonstrated that reductants, SOD, CAT and BSA could protect MN9D cells against DA, AM or H_2O_2-induced toxicity with different profiles. GSH at 200 μM could completely abrogate the cell viability decrease induced by 100 or 200 μM DA overnight challenges (Figure 6A). However, 400 μM AA or 1 mM Vitamin E could only partially abrogate cell viability decrease induced by 200 μM DA (Figure 6A). The DA-induced cell viability decrease could also be partially alleviated by 500 unit/ml SOD, CAT and by 25 mg/ml BSA (Figure 6B). However, lower concentration of BSA (0.125 mg/ml, corresponding to the protein amounts of 500 unit/ml CAT or SOD) could not significantly alleviate AM induced cell death (data not shown). On the other hand, 200 or 400 μM GSH could significantly prevent 50–200 μM AM-induced cell death in a dosage dependent manner, while 400 and 800 μM AA could have poor protective effect against 50–200 μM AM-induced cell death (Figure 6C). No significant protective effect by Vitamin E against AM-induced cell death could be observed (Figure 6C). The AM-induced cell death could not be alleviated by 500 unit/ml SOD or CAT but could be significantly

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

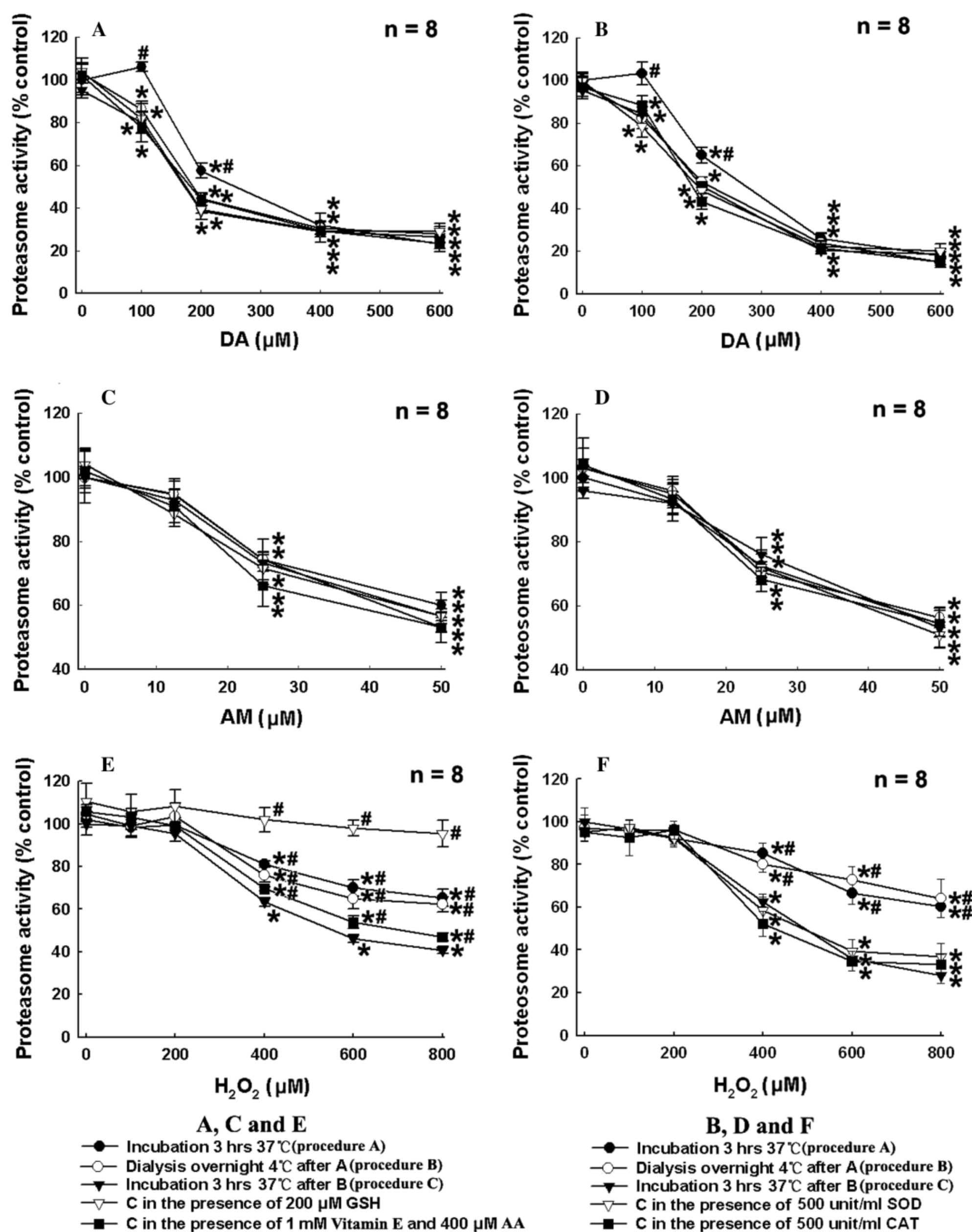


Figure 5. Reversion of DA, AM or H_2O_2 induced inhibition of chymotrypsin-like proteasome activity by GSH, SOD, CAT, Vitamin E and AA. Cell extracts of MN9D cells in buffer A were incubated with DA (A and B), AM (C and D) or H_2O_2 (E and F) at 37°C for 3 h (we called this procedure A), then the cell extracts were dialysed against buffer A overnight at 4°C in dialysis tube (MWCO 100KD) with at least two changes of buffer A after procedure A (we called this procedure B). The dialysed cell extracts were further incubated at 37°C for 3 h with or without GSH, SOD, CAT, Vitamin E and AA after procedure B (we called this procedure C). Finally proteasome substrate was added and cell extracts were further incubated for 30 min at 37°C before proteasome activity detection. Proteasome activity was shown as '% control'. Proteasome activities of cell extracts without challenge were as 'controls' (* at least $p < 0.05$, compared with controls; # at least $p < 0.05$, compared with those challenged with same concentration of DA, AM or H_2O_2 only in procedure C).

alleviated by 25 mg/ml BSA (Figure 6D). We demonstrated that 1 mM Vitamin E or 200 μM GSH could alleviate cell death caused by 20 or 50 μM H_2O_2 challenge overnight in a H_2O_2 dosage dependent manner (Figure 6E). AA had no effect on the cell viability decrease caused by H_2O_2 (Figure 6E). We also demonstrated that 20 or 50 μM H_2O_2-induced cell death could be completely alleviated by 500 unit/ml CAT but could not be influenced by 500 unit/ml SOD or 25 mg/ml BSA (Figure 6F).

SOD and CAT could alleviate DA auto-oxidation in buffer A

We further demonstrated that SOD and CAT could significantly abrogate DA auto-oxidation in buffer A by HPLC analysis (Figure 7). After 3 h incubation in buffer A at 37°C, the peak areas of 200 and 400 μM DA decrease significantly (Figure 7). However, in the presence of 500 and 1000 unit/ml SOD or CAT, the decreased DA peak areas could be significantly reserved (Figure 7). However, 25 mg/ml BSA had

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

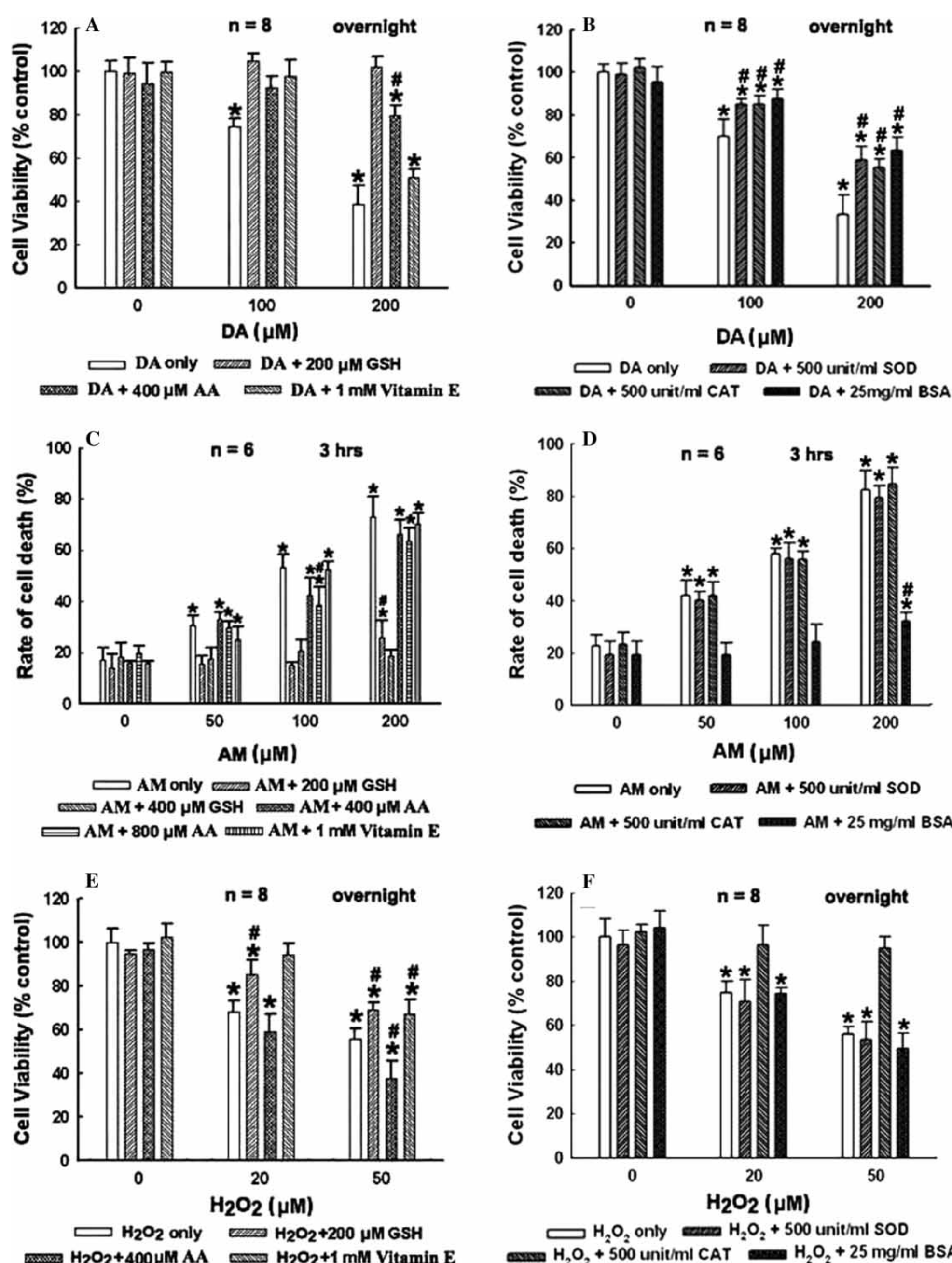


Figure 6. Alleviation of MN9D cell death induced by DA, AM or H_2O_2 by GSH, SOD, CAT, Vitamin E, AA and 25 mg/ml BSA. MN9D cells were challenged with DA (A and B), AM (C and D) or H_2O_2 (E and F) in the presence or absence of GSH, SOD, CAT, Vitamin E, AA and 25 mg/ml BSA overnight or for 3 h. Viability of MN9D cells were detected by MTT assay and shown as '% control' or by trypan blue staining and expressed as 'rate of cell death (%)'. For AM challenge (C and D), DMEM medium was changed to PBS just before administering AM to cells. Then freshly prepared AM was added. AM was replenished every hour. Cells without challenge were as 'controls' (* at least $p < 0.05$, compared with respective controls; # at least $p < 0.05$, compared with cells challenged with same concentration of DA, AM or H_2O_2 only but without by GSH, SOD, CAT, Vitamin E, AA and 25 mg/ml BSA).

no effect on DA auto-oxidation process in buffer A (Figure 7).

BSA could absorb AM and abrogate melanin formation in solutions

We further demonstrated that, in the presence of 25 mg/ml BSA, the aggregation of AM and formation of melanin was abrogated (Figure 8). In the absence of 25 mg/ml BSA, incubation of 50 and 100 μM AM in buffer A for 3 h could lead to AM aggregation and formation of melanin, which showed a monotonous increase of light absorbance within all ranges of wavelength (Figure 8A and B). In the presence of 25 mg/ml BSA, the AM specific light absorbance peak at 465 nm wavelength as well as monotonous increase of light absorbance disappeared after incubation, indicating absorbance of AM and disruption of

RIGHTSLINK

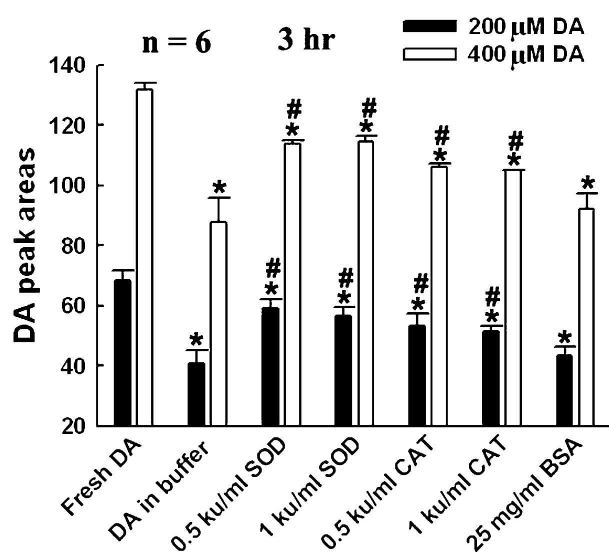


Figure 7. Abrogation of DA auto-oxidation in solution by SOD, CAT and 25 mg/ml BSA; 200 and 400 μM freshly prepared DA was incubated in buffer A in the presence or absence of SOD, CAT and 25 mg/BSA at 37°C for 3 h and then analysed by HPLC procedure at 280 nm absorbance. Peak areas of DA before and after incubation was monitored (* at least $p < 0.05$, compared with peak areas of freshly prepared DA, # at least $p < 0.05$, compared with peak areas of DA in the absence of SOD, CAT or BSA).

melanin formation by BSA after incubation (Figure 8A and B). Similar phenomenon could also be detected when 200 or 800 μM DA had been incubated in buffer A for 3 h in the presence of 25 mg/ml BSA (Figure 8C and,D). The monotonous increase of light absorbance within whole range of wavelength disappeared (for 200 μM DA) or significantly alleviated (for 800 μM DA) after DA had been incubated in buffer A in the presence of 25 mg/ml BSA, suggesting absorbance of AM derived from DA auto-oxidation and disruption of melanin formation by high concentration of BSA (Figure 8C and D). However, low concentration BSA (0.125 mg/ml) did not have these effects above-mentioned (data not shown).

Discussion

DA oxidation could produce reactive DA quinones as well as small molecular weight ROS [3]. In this study we revealed for the first time that DA as well as its oxidative metabolites AM (cyclized DA quinone) and H_2O_2 could all directly inhibit proteasome activity or cooperate with proteasome inhibitor MG132 to induce aggravated proteasome inhibition in a dosage and/or incubation time-dependent manner. The proteasome inhibition induced by DA was intensive and could be alleviated by GSH, AA, Vitamin E, SOD and CAT, especially by GSH, but was irreversible. The proteasome inhibition induced by AM was also intensive, irreversible and could be efficiently

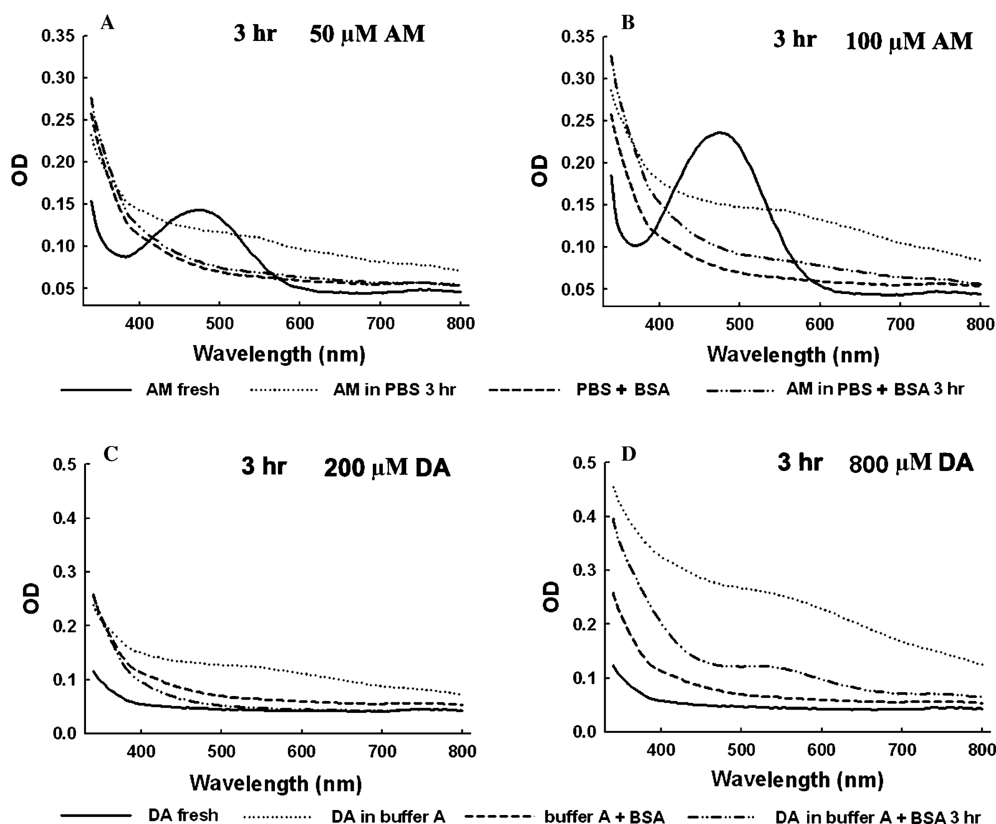


Figure 8. BSA could absorb AM and abrogate melanin formation in solutions. (A) 50 μM AM; (B) 100 μM AM, (C), 200 μM DA and (D) 800 μM DA freshly prepared or incubated in PBS or buffer A with or without 25 mg/ml BSA at 37°C for 3 h were scanned and analysed by spectrometer. Change of light absorbance of continuous wavelength were recorded and demonstrated.

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

preventable by GSH but could not be alleviated by Vitamin E, SOD and CAT. On the other hand, the proteasome inhibition by H_2O_2 was weaker than those by DA or AM and was preventable by GSH, AA plus Vitamin E and CAT but was reversible by GSH. Taken all together, we concluded that DA-derived DA quinones, such as AM, rather than small molecular weight ROS, such as H_2O_2, could account for the intensive and irreversible proteasome inhibition induced by DA. The malfunction of UPS induced by exopathic or idiopathic factors has been supposed to be a universal point underlying both the familial and sporadic forms of PD [11,26]. Therefore, DA and its oxidative metabolites, especially DA-derived quinones, could be considered as endogenous UPS inhibitors, which could impair UPS function and cell viability of dopaminergic neurons and finally contribute to selective dopaminergic neurodegeneration of PD.

We demonstrated in this study that SOD and CAT could abrogate DA-induced proteasome inhibition and cell toxicity but could not alleviate AM-induced proteasome inhibition and cell toxicity. SOD and CAT could both alleviate DA auto-oxidation in solutions. It has been reported that the process of DA auto-oxidation involved initiation, propagation and termination steps, whereas the ROS produced in the process of DA initiation step was important for further propagation steps of DA auto-oxidation [27]. Hereby we proposed that SOD and CAT could scavenge ROS produced in the process of DA auto-oxidation and therefore interrupt DA auto-oxidation and thus could alleviate DA-induced proteasome inhibition and cell toxicity. Our recent study demonstrated that DA could auto-oxidize to produce AM while GSH, AA and Vitamin E, especially GSH, could significantly inhibit DA auto-oxidation and thus abrogate AM production [25]. Therefore reductants, SOD and CAT could function to abrogate DA auto-oxidation and the subsequent production of DA metabolites. They thus could alleviate DA-induced irreversible proteasome inhibition and even dopaminergic cell degeneration.

Our conclusion that the toxicity of AM to cells was stronger than that of H_2O_2 conformed to recent findings that highly reactive DA-derived quinones rather than ROS play an important role in DA-induced toxicity [6–8]. DA quinones could react with sulphydryl groups containing chemicals including the cysteine residuals of functional proteins in dopaminergic cells [5]. However, the cross-linking and irreversible inactivation of functional proteins in dopaminergic cells by DA quinones could be prevented by a GSH-related detoxification mechanism. GSH could provide its sulphydryl groups to compete with those of functional proteins to covalently conjugate with DA quinones and thus detoxify toxic DA quinones. In the current study, we demonstrated that GSH was the only potent agent among all protective agents to protect against AM-induced proteasome inhibition and cell toxicity. This was consistent with our previous findings that GSH could provide its sulhhydryl groups and react with AM to form various GSH-AM conjugations and thus could function as DA quinones quenchers [25].

In this study, we also demonstrated that high concentration of BSA (25 mg/ml) could significantly protect MN9D cells against AM-induced cell death, whereas low concentration of BSA could not (25 mg/ml BSA could provide $\sim$10 mM cysteine residues for AM conjugation). Therefore high concentration of BSA should be able to provide sufficient cysteine residues to conjugate with AM and function to protect MN9D cells against AM-induced toxicity. The protective mechanism of high concentration of BSA against AM-induced toxicity could be further supported by our findings demonstrating that high concentration of BSA could absorb AM and abrogate AM aggregation and melanin formation. Our findings implicated the toxic mechanism of AM in relation to dopaminergic neuron degeneration as well as protective mechanisms of GSH to detoxify AM-induced toxicity. Post-mortem studies demonstrated that there was an obvious decrease ($\sim$50%) of GSH in SN of PD patients compared with aged controls, which was not accompanied by a corresponding increase in glutathione disulphide (GSSG) [28,29]. The decrease of GSH level of SN in PD patients was found to be also not due to decreased activities of enzymes for biosynthesis or metabolism of GSH [28]. Furthermore, there was a significant increase of conjugates of cysteine or GSH with DA quinones in SN of PD patients [30]. These data implicated the irreversible consumption of GSH and decrease of GSH level in SN of PD patients due to conjugation and detoxification of DA quinones by GSH, which may actually play an important role in PD pathogenesis.

However, GSH could not be transported through the blood–brain barrier to reach the dopaminergic neurons, therefore suitable approaches should be applied to increase cellular GSH syntheses *in vivo* or introduce sulphydryl containing GSH substitutes into SN to complement the protective function of GSH in dopaminergic neurons [31]. N-acetylcysteine (NAC) has been proposed to treat age-associated neurodegenerative diseases, such as PD [32]. NAC was found to prevent 6-hydroxydopamine-induced programmed cell death in cultured neuronal cells [33]. NAC was also found to improve mitochondrial activities of aged mice [34]. These effects could be ascribed to the chemical properties of NAC. First, NAC could act as a precursor for GSH synthesis and a stimulator of enzymes involved in GSH regeneration [32]. Secondly, NAC was also a reductant and could scavenge ROS. Furthermore, NAC contains

RIGHTSLINK

sulphydryl group and could covalently react with DA quinones with its thiol group to detoxify reactive DA quinones [32]. Our recent study had demonstrated that NAC could rescue dopaminergic PC12 cell death induced by over-expression of mutant human A53T alpha-synuclein via increasing the total and reduced GSH levels of PC12 cells [35]. In view of the above and because of the ease of its administration as well as non-toxicity in humans, we propose the use of NAC in therapy to control PD onset and development, for sporadic as well as for the familial form of PD patients.

Based on findings in this study, we further propose that dopaminergic neurons in the *substantia nigra* (SN) may have at least two protective mechanisms against DA-induced toxicity. The first is to inhibit DA oxidation by reductants and ROS-related enzymes; and the second is to eliminate DA oxidative metabolites in time, especially DA-derived quinones. The elimination of DA oxidative metabolites include scavenging of ROS by reductants and by ROS-related enzymes as well as detoxification of DA-derived quinones by quinone conjugating reagents, such as GSH or quinines-related enzymes. *Vice versa*, factors to trigger or accelerate DA oxidation in dopaminergic neurons would be vital to PD pathogenesis. To date, iron species have been implicated to be highly related to PD pathogenesis [36]. It was demonstrated that there was a decrease in GSH level associated with an increase of iron level in the degenerating SN of PD patients [37]. Our unpublished data demonstrated that iron species could significantly mediate DA oxidation, increase DA quinones production and subsequently lead to proteasome inhibition and dopaminergic cell demise. However these toxicities of iron species could be alleviated by GSH and iron chelators via different mechanisms. These data implicated that iron species might contribute to PD pathogenesis via triggering and acceleration of DA oxidation as well as increase production of reactive DA metabolites. Our recent findings demonstrated that auto-oxidation of endogenous DA could aggravate over-expression of mutant human A53T alpha-synuclein induced PC12 cell death [34]. Taken together, it is logical to infer that accelerated DA oxidation and increased DA metabolites production might be the converging events linking various deleterious factors related to PD pathogenesis. Consequently, agents such as NAC which are effective in controlling DA oxidation and elimination of DA metabolites should be able to control PD onset and development.

Acknowledgements

This project was supported by a grant from the Biomedical Research Council, A*STAR, Singapore. We thank Dr Alfred Heller (University of Chicago) for the use of MN9D cells.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Lotharius J, Brundin P. Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. Nat Rev Neurosci 2002;3:932–942.

[2] Bisaglia M, Mammi S, Bubacco L. Kinetic and structural analysis of the early oxidation products of dopamine: analysis of the interactions with alpha-synuclein. J Biol Chem 2007; 282:15597–15605.

[3] Graham DG, Tiffany SM, Bell WR Jr, Gutknecht WF. Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells *in vitro*. Mol Pharmacol 1978;14:644–653.

[4] Kuhn DM, Arthur R Jr. Dopamine inactivates tryptophan hydroxylase and forms a redox-cycling quinoprotein: possible endogenous toxin to serotonin neurons. J Neurosci 1998; 18:7111–7117.

[5] Xu Y, Stokes AH, Roskoski R Jr, Vrana KE. Dopamine, in the presence of tyrosinase, covalently modifies and inactivates tyrosine hydroxylase. J Neurosci Res 1998;54:691–697.

[6] Izumi Y, Sawada H, Yamamoto N, Kume T, Katsuki H, Shimohama S, Akaike A. Iron accelerates the conversion of dopamine-oxidized intermediates into melanin and provides protection in SH-SY5Y cells. J Neurosci Res 2005;82:126–137.

[7] Asanuma M, Miyazaki I. Nonsteroidal anti-inflammatory drugs in Parkinson's disease: possible involvement of quinone formation. Expert Rev Neurother 2006;6:1313–1325.

[8] Kobayashi M, Kim J, Kobayashi N, Han S, Nakamura C, Ikebukuro K, Sode K. Pyrroloquinoline quinone (PQQ) prevents fibril formation of alpha-synuclein. Biochem Biophys Res Commun 2006;349:1139–1144.

[9] Berke SJ, Paulson HL. Protein aggregation and the ubiquitin proteasome pathway: gaining the UPPer hand on neurodegeneration. Curr Opin Genet Dev 2003;13:253–261.

[10] McNaught KS, Mytilineou C, Jnobaptiste R, Yabut J, Shashidharan P, Jennert P, Olanow CW. Impairment of the ubiquitin-proteasome system causes dopaminergic cell death and inclusion body formation in ventral mesencephalic cultures. J Neurochem 2002;81:301–306.

[11] McNaught KS, Olanow CW, Halliwell B, Isacson O, Jenner P. Failure of the ubiquitin-proteasome system in Parkinson's disease. Nat Rev Neurosci 2001;2:589–594.

[12] Benbunan BR, Korczyn AD, Giladi N. Parkin mutation associated parkinsonism and cognitive decline, comparison to early onset Parkinson's disease. J Neural Transm 2004; 111:47–57.

[13] Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, Harta G, Brownstein MJ, Jonnalagada S, Chernova T, Dehejia A, Lavedan C, Gasser T, Steinbach PJ, Wilkinson KD, Polymeropoulos MH. The ubiquitin pathway in Parkinson's disease. Nature 1998;395:451–452.

[14] Farrer M, Chan P, Chen R, Tan L, Lincoln S, Hernandez D, Forno L, Gwinn-Hardy K, Petrucelli L, Hussey J, Singleton A, Tanner C, Hardy J, Langston JW. Lewy bodies and parkinsonism in families with parkin mutations. Ann Neurol 2001;50:293–300.

[15] Zafar KS, Siegel D, Ross D. A potential role for cyclized quinones derived from dopamine, DOPA, and 3,4-dihydroxyphenylacetic acid in proteasomal inhibition. Mol Pharmacol 2006;70:1079–1086.

[16] Bazzini E, Samuele A, Granelli M, Levandis G, Armentero MT, Nappi G, Blandini F. Proteasomal inhibition and

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

RIGHTSLINK

apoptosis regulatory changes in human isolated lymphocytes: the synergistic role of dopamine. J Cell Biochem 2007; 8;103:877–885.
[17] Yoshimoto Y, Nakaso K, Nakashima K. L-dopa and dopamine enhance the formation of aggregates under proteasome inhibition in PC12 cells. FEBS Lett 2005;579:1197–1202.
[18] Keller JN, Huang FF, Dimayuga ER, Maragos WF. Dopamine induces proteasome inhibition in neural PC12 cell line. Free Radic Biol Med 2000;29:1037–1042.
[19] Zhou Z, Kerk S, Meng Lim T. Endogenous dopamine (DA) renders dopaminergic cells vulnerable to challenge of proteasome inhibitor MG132. Free Radic Res 2008;42:456–466.
[20] Mytilineou C, McNaught KS, Shashidharan P, Yabut J, Baptiste RJ, Parnandi A, Olanow CW. Inhibition of proteasome activity sensitizes dopamine neurons to protein alterations and oxidative stress. J Neural Transm 2004;111:1237–1251.
[21] Choi HK, Won LA, Kontur PJ, Hammond DN, Fox AP, Wainer BH, Hoffmann PC, Heller A. Immortalization of embryonic mesencephalic dopaminergic neurons by somatic cell fusion. Brain Res 1991;552:67–76.
[22] Dick LR, Cruikshank AA, Destree AT, Grenier L, McCormack TA, Melandri FD, Nunes SL, Palombella VJ, Parent LA, Plamondon L, Stein RL. Mechanistic studies on the inactivation of the proteasome by lactacystin in cultured cells. J Biol Chem 1997;272:182–188.
[23] Rao S, Porter DC, Chen X, Herliczek T, Lowe M, Keyomarsi K. Lovastatin-mediated G1 arrest is through inhibition of the proteasome, independent of hydroxymethyl glutaryl-CoA reductase. Proc Natl Acad Sci USA 1999;96:7797–7802.
[24] Braun HA, Umbreen S, Groll M, Kuckelkorn U, Mlynarczuk I, Wigand ME, Drung I, Kloetzel PM, Schmidt B. Tripeptide mimetics inhibit the 20 S proteasome by covalent bonding to the active threonines. J Biol Chem 2005;280:28394–28401.
[25] Zhou ZD, Lim TM. Roles of glutathione (GSH) in dopamine (DA) oxidation studied by improved tandem HPLC plus ESI-MS. Neurochem Res 2009;34:316–326.
[26] McNaught KS, Belizaire R, Jenner P, Olanow CW, Isacson O. Selective loss of 20S proteasome alpha-subunits in the substantia nigra pars compacta in Parkinson's disease. Neurosci Lett 2002;326:155–158.

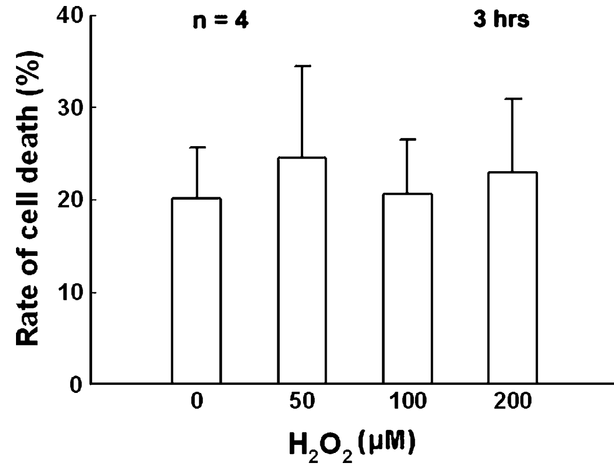
This paper was first published online on iFirst on 17 March 2009.

[27] Klegeris A, Korkina LG, Greenfield SA. Autoxidation of dopamine: a comparison of luminescent and spectrophotometric detection in basic solutions. Free Radic Biol Med 1995;18:215–222.
[28] Jenner P, Dexter DT, Sian J, Schapira AH, Marsden CD. Oxidative stress as a cause of nigral cell death in Parkinson's disease and incidental Lewy body disease. The Royal Kings and Queens Parkinson's Disease Research Group. Ann Neurol 1992;32(Suppl):S82–S87.
[29] Spencer JP, Jenner P, Halliwell B. Superoxide-dependent depletion of reduced glutathione by L-DOPA and dopamine. Relevance to Parkinson's disease. Neuroreport 1995;6:1480–1484.
[30] Spencer JP, Jenner P, Daniel SE, Lees AJ, Marsden DC, Halliwell B. Conjugates of catecholamines with cysteine and GSH in Parkinson's disease: possible mechanisms of formation involving reactive oxygen species. J Neurochem 1998;71:2112–2122.
[31] Piga R, Saito Y, Yoshida Y, Niki E. Cytotoxic effects of various stressors on PC12 cells: involvement of oxidative stress and effect of antioxidants. Neurotoxicology 2007;28:67–75.
[32] Banaclocha MM. Therapeutic potential of N-acetylcysteine in age-related mitochondrial neurodegenerative diseases. Med Hypotheses 2001;56:472–477.
[33] Munoz AM, Rey P, Soto-Otero R, Guerra MJ, Labandeira-Garcia JL. Systemic administration of N-acetylcysteine protects dopaminergic neurons against 6-hydroxydopamine-induced degeneration. J Neurosci Res 2004;76:551–562.
[34] Martinez Banaclocha M. N-acetylcysteine elicited increase in complex I activity in synaptic mitochondria from aged mice: implications for treatment of Parkinson's disease. Brain Res 2000;859:173–175.
[35] Zhou ZD, Kerk SY, Xiong GG, Lim TM. Dopamine autooxidation aggravates non-apoptotic cell death induced by overexpression of human A53T mutant alpha-synuclein in dopaminergic PC12 cells. J Neurochem 2009;108:601–610.
[36] Salazar J, Mena N, Nunez MT. Iron dyshomeostasis in Parkinson's disease. J Neural Transm Suppl 2006;205–213.
[37] Bharath S, Hsu M, Kaur D, Rajagopalan S, Andersen JK. Glutathione, iron and Parkinson's disease. Biochem Pharmacol 2002;64:1037–1048.

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

RIGHTSLINK

Supplementary materials



Supplementary Figure. Exposure to H_2O_2 for 3 h did not induce more cell death in MN9D cells (Supplementary data to Figure 6). Challenging MN9D cells with 50–200 μM H_2O_2 for 3 h duration could not induce significant cell death. Challenge of cells with H_2O_2 and cell death detection was exactly similar to AM experiments in Figure 6(C and D). This set of data serves to compare with the effect of AM exposure for 3 h shown in Figure 6, to verify that indeed AM is more toxic than H_2O_2.

Free Radic Res Downloaded from informahealthcare.com by Newcastle University on 12/04/11
For personal use only.

RIGHTSLINK