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Compromised proteasome degradation elevates neuronal nitric oxide synthase levels and induces apoptotic cell death

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Abstract

The significance of impairment of proteasome activity in PC12 cells was examined in connection with nitrative/nitrosative stress and apoptotic cell death. Treatment of differentiated PC12 cells with MG132, a proteasome inhibitor, elicited a dose- and time-dependent increase in neuronal nitric oxide synthase (nNOS) protein levels, decreased cell viability, and increased cytotoxicity. Viability and cytotoxicity were ameliorated by L-NAME (a broad NOS inhibitor). Nitric oxide/peroxynitrite formation was increased upon treatment of PC12 cells with MG132 and decreased upon treatment with the combination of MG132 and 7-NI (a specific inhibitor of nNOS). The decreases in cell viability appeared to be effected by an activation of JNK and its effect on mitochondrial Bcl-x_L phosphorylation. These effects are strengthened by the activation of caspase-9 along with increased caspase-3 activity upon treatment of PC12 cells with MG132. These results suggest that impairment of proteasome activity and consequent increases in nNOS levels lead to a nitrative stress that involves the coordinated response of JNK cytosolic signaling and mitochondrion-driven apoptotic pathways.

Keywords

nNOS; nitration; UPS; apoptosis; PC12 cells; caspase-9; nitric oxide; nitrite; proteasome

Introduction

The degradation of short-lived and oxidized proteins is the domain of the ubiquitin-proteasome system (UPS): impairment of proteasome activity occurs during aging and in a variety of age-related neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease [1–3]. The ubiquitin-proteasome system—an intracellular multicatalytic protease—is (a) present in all cell types in the central nervous system [4], (b) responsible for the majority of intracellular proteolysis of oxidized, misfolded, and aggregated proteins [5–7], and (c) itself susceptible to free radical attack; also some proteins damaged by free radicals may as well impair the function of UPS [8–10]. A compromised UPS leads to accumulation of abnormal proteins [11,12], decrease in GSH levels, and increase in protein oxidation. In the case of Parkinson's disease, the formation of ubiquitinated inclusions of misfolded proteins known as Lewy bodies suggests a compromised ubiquitin-proteasome degradation system.

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One of the well-known degradation targets of the UPS are nitric oxide synthases (NOS) [13–16], isoenzymes that catalyze the five-electron oxidation of the guanidine group of L-arginine with formation of nitric oxide (NO) [17,18]. NOS activity increases during aging in various parts of brain, such as hippocampus, cortex, and cerebellum [19–21]. The identity of the NOS isoform contributing to the higher NO production in the aging brain is a matter of debate [19–22]. However, neuronal NOS (nNOS) – a Ca^{2+} -calmodulin-dependent isoform of NOS that is constitutively expressed in neuronal cells [23] – is suggested as the isoform responsible for the higher NO production during normal aging, whilst iNOS may be involved in age-associated brain pathologies [20].

The role of NO produced by NOS in cell fate is diverse and probably dependent on concentration gradients and target proximity: an anti-apoptotic function of NO is based on the nitrosylation and consequent inhibition of caspases thereby preventing apoptotic cell death [24]. Conversely, the pro-apoptotic actions of NO are probably based on the inhibition of cytochrome oxidase, thereby lowering the mitochondrial membrane potential and activation of the permeability transition pore [25,26]. NO -derived species, such as peroxynitrite (ONOO^-) (the product of the diffusion-controlled reaction between NO and $\text{O}_2^{\cdot-}$) are also involved in regulation of apoptotic pathways: for example, the direct damage on DNA by ONOO^- causes up-regulation of the pro-apoptotic protein Bax and eventually activation of the apoptotic cascade [27,28].

In this study, we examine the effects of NO caused by an accumulation of nNOS protein levels due to compromised UPS, thereby addressing two major concepts in age-related neurodegenerative disorders, oxidative/nitrosative stress and impairment of the UPS system and the encompassing redox-sensitive signaling pathways in PC12 cells associated with apoptotic cell death. Experiments were performed on PC12 cells, a model for dopaminergic cells with well-defined steps triggered by apoptotic stimuli (in response to metabolic, oxidative stress-, or apoptotic signals). Hence, an advantage of the PC12 model over different neuronal cell lines is that the response of the latter to different stress stimuli is markedly different.

Materials and methods

Materials and cell culture

Antibodies against nNOS was purchased from Sigma (St Louis, MO); JNK1, pJNK and caspase-9 were purchased from Santa Cruz Biotech (Santa Cruz, CA); β -actin was from Chemicon (Temecula, CA); p-Bcl-x_L was from Upstate (Lake Placid, NY). The phosphatase inhibitor cocktail (Catalog № 524624; Calbiochem, La Jolla, CA) contained (-)-p-bromotetramisole oxalate, cantharidin, and microcystin LR. The protease inhibitor cocktail (catalog № P8340) and all other chemicals or reagents were obtained from Sigma (St Louis, MO). Rat pheochromocytoma cells (PC12) were purchased from ATCC and maintained in growth medium (RMPI + 10% donor horse serum + 5% fetal bovine serum). Cells differentiation was assessed in differentiation medium (RPMI medium + 1% donor horse serum + 100 ng NGF/ml + 50 ng cAMP/ml) for 5 days.

Cell viability and cytotoxicity measurements

Differentiated PC12 cells were cultured in 6-well plates at 7×10^6 cells/well and treated with MG132 (0 to 10 μM) in differentiation medium for 24- or 48 h and then subject to MTT or LDH assay. For rescue experiments, cells were pretreated with 3 mM L-NAME for 30 min prior to MG132 treatment. Cell viability was assessed by incubating treated cells with MTT (5 mg/ml) (Sigma, St Louis, MO) for 30 min at 37°C in a humidified 5% CO_2 incubator. The reduced intracellular formazan product was dissolved by replacing 2 ml of differentiation medium with the same volume of DMSO. The absorbance at 490 nm was measured with a

microplate reader. Data are expressed as the percentages of cell viability calculated by the following equation: cell viability = $(OD_{\text{treatment group}}/OD_{\text{control group}}) \times 100\%$. Cytotoxicity was evaluated as lactate dehydrogenase release (Promega, Madison, WI).

Proteasome inhibition assay

PC12 cells were disrupted by sonication in buffer containing: 10 mM HEPES, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4 , 5 mM ATP, 5 mM MgCl_2 , 1 mM DTT, and 10% glycerol, at pH 7.4. Proteasome activity was assayed on 100 μg cell lysate, measured as 20S proteasome activity (Chemicon, Temecula, CA), and quantified by the fluorescence originating from the cleavage of proteasome substrate (LLVY-AMC) using a 380/460 nm filter set and expressed as percentage inhibition of the control sample.

Nitrite determination with 2,3-diaminonaphthalene (DAN method)

NO production was measured as total NO_2^- present in the cell medium and detected by the DAN assay [29]. Briefly, PC12 cells were incubated in HEPES buffer containing NGF and cAMP in order to prevent interference caused by high level of NO_2^- present in the RPMI. Cells were exposed to MG132 (0.5 μM) at 37°C for 24 h; 10 μl freshly prepared DAN (0.5 mg/ml in 0.62 M HCl) was added to 1 ml of sample or positive control and stirred immediately in the reaction buffer containing 40 μM NADPH with or without nitrate reductase (10 units/ml). After 30 min incubation at 37°C in the dark, the reaction was terminated by 50 μl of 2.8 N NaOH. Formation of 1-H-naphthotriazole was measured with a fluorescence spectrophotometer (Molecular Devices, SpectraMax, USA) with excitation wavelength 365 nm and emission wavelength 450 nm.

Caspase-9 and caspase-3 measurements

Activation of caspase-9 was assessed by western blot using anti-caspase 9 antibody to detect the appearance of active caspase-9 (cleaved form) with different doses of either MG132 or MG132 + L-NAME. Caspase-3 activity was measured colorimetrically (Promega, Madison, WI): untreated cells or cells treated with either MG132 (0.5 μM) or pretreated with L-NAME (3 mM) for 20 min and then supplemented with MG132 (0.5 μM) were incubated with Ac-DEVD-pNA for 24 h at 22°C. The release of chromophore *p*-nitroaniline was quantified by a absorbance at 405 nm. Z-VAD-FM- treated cells were included as negative control.

Statistical analysis

Data are reported as means $\pm$ S.D. of at least three independent experiments. Significant differences between mean values were determined by student *t*-test. Means were considered to be statistically distinct if $P < 0.05$.

Results

Inhibition of proteasome activity leads to elevated nNOS content in PC12 cells

Differentiated PC12 were treated with different concentrations of MG132 for 24- and 48 h (Fig. 1). Total proteins were harvested and analyzed by western blot using an antibody against the N-terminal of nNOS. nNOS protein levels were increased exponentially when incubated for 24 h with different amounts of the proteasome inhibitor, MG132. More than one nNOS band was observed at high concentrations ($> 0.5 \mu\text{M}$) of- and prolonged (48 h) treatment with MG132: this may be due to a partial degradation of nNOS proteins or the occurrence of nNOS splicing variants in PC12 cells, for the C-terminal targeting antibody did not detect nNOS in the current experimental model. iNOS and eNOS proteins were not detected in PC12 cells (data not shown).

Increase in nNOS protein content leads to increase in cytotoxicity and decrease in neuronal viability

Increased nNOS content leads to increased nitrate stress, thus causing progressive neuronal cell death by apoptotic and/or necrotic events. Treatment of differentiated PC12 cells with different concentrations of MG132 for 24 h resulted in a dose-dependent decline of PC12 viability (Fig. 2A). Pretreatment of PC12 cells with L-NAME—a broad NOS inhibitor—partially rescued MG132-induced cell viability, thus suggesting the involvement of nNOS and NO in the events leading to decreased cell viability. Cytotoxicity (LDH release) was also affected by treatment with increasing concentrations of MG132 (Fig. 2B); as above, cytotoxicity was far less pronounced when PC12 cells were pretreated with L-NAME. Fig. 2C shows that the extent of proteasome inhibition by different concentrations of MG132 was inversely associated with a decrease in cell viability. In summary, data in Fig. 2 support the notion that increases in nNOS levels due to proteasome inhibition are involved in decreased cell viability and increased cytotoxicity.

Accumulation of nNOS protein leads to enhanced NO production in PC12 cells

NO production was assessed as nitrite (NO_2^-) levels in the medium measured by DAN method in the absence and presence of nitrate reductase, an enzyme that converts NO_3^- to NO_2^- . PC12 cells treated with $0.5 \mu\text{M}$ of MG132 in HEPES buffer for 24 h showed no significant increases in NO_2^- production (Fig. 3A) in the absence of nitrate reductase; however, specific inhibition of nNOS by 7NI resulted in 45% decrease in NO_2^- production. In the presence of nitrate reductase, total nitrite levels ($\text{NO}_2^- + \text{NO}_3^-$) were increased by 45% upon treatment of PC12 cells with MG132 (Fig. 3B). The involvement of nNOS in NO_2^- production is supported by the strong inhibitory effect of 7-NI. It is noteworthy that the total nitrite levels ($\text{NO}_2^- + \text{NO}_3^-$) (measured in the presence of nitrate reductase; Fig. 3B) are one order of magnitude higher than NO_2^- levels measured in the absence of nitrate reductase (Fig. 3A). This suggests that a major source of NO_2^- originates from the reduction of NO_3^- formed upon peroxynitrous acid (ONOOH) isomerization; minor sources of NO_2^- can be ascribed to N_2O_3 hydration to HNO_2 (followed by deprotonation to NO_2^-) and likely reactions of peroxynitrous acid with nucleophiles (e.g., $\text{RS}^- + \text{ONOOH} \rightarrow \text{RSOH} + \text{NO}_2^-$) or two electron reduction of ONOO^- by metalloporphyrins.

Increased ONOO^- production activated JNK signaling pathway and decreased cell viability

Levels of active (phosphorylated) JNK, a stress response MAPK, were measured by immunoblotting utilizing an antibody against bisphosphorylated JNK (p-JNK). Treatment of PC12 cells with $0.5 \mu\text{M}$ MG132 for 24 h resulted in increased levels of active (phosphorylated) JNK (Fig. 4A). JNK activation appears secondary to- and dependent on increased nNOS activity; this notion is supported by the strong inhibitory effect on pJNK levels of 7-NI, a specific nNOS inhibitor. As expected, JNK activation upon treatment with MG132 was partially inhibited by SP600125, a rather specific JNK inhibitor. These data suggest that reactive nitrogen species, formed by elevated levels of nNOS upon proteasome inhibition, are involved in the activation of JNK, thus confirming previous reports [30]. It has been shown that upon activation, JNK1 translocates to mitochondria and triggers a phosphorylation cascade in these organelles [31,32]; Bcl-2 and Bcl-x_L are among the outer mitochondrial membrane proteins phosphorylated (and inactivated) upon translocation of JNK to the organelles: as shown in Fig. 4A, inhibition of proteasome activity in PC12 cells resulted in phosphorylation of the constitutive Bcl-x_L in mitochondria; this phosphorylation was decreased upon inhibition of nNOS and JNK in MG132-treated PC12 cells. Measurements of PC12 cell viability followed the pattern in Fig. 4A: it was decreased by approximately 50% upon treatment with MG132 and partially rescued upon inhibition of nNOS and of JNK activation (Fig. 4B).

JNK signaling leads to necrotic and apoptotic cell death

In line with the increased Bcl-x_L phosphorylation observed in Fig. 4A, treatment of PC12 cells with different concentrations of MG132 for 24 h resulted in a dose-dependent increase of active caspase-9 (Fig. 5A), which is involved in the mitochondrion-driven apoptotic pathway. Cleavage of caspase-9 upon MG132 treatment was sensitive to NOS inhibition by L-NAME (Fig. 5B). Activity of caspase-3, the executor caspase for both the intrinsic- and extrinsic apoptotic pathways, was increased upon treatment with MG132 (0.5 μM for 24 h); this activity was decreased by 60–70% upon inhibition of NOS (with either L-NAME or 7-NI). Z-VAD-FMK, which inhibits caspase-3, was included as a negative control for MG132-treated PC12 cells and control cells.

Discussion

Parkinson's disease is characterized by loss of dopaminergic neurons accompanied with a compromised proteasome degradation system which leads to formation of Lewy bodies; this, together with the increasing evidence of age-related decline of proteasome activity and increase of nNOS protein levels, validate the current PC12 cell model as an approach of age-related neurodegenerative disorders. Other features of the PC12 model of relevance to this study are (a) the ability of dopamine to inhibit the proteasome system [33], (b) the ability of PC12 cells to express both JNK1 and -2 isoforms, and (c) the high sensitivity of PC12 cells to undergo apoptosis in response to both H₂O₂ and TNF-α [34]. Furthermore, PC12 cells were used as a model to assess mitochondrial function *in situ* [35].

Dose-dependent inhibition of UPS in differentiated PC12 cells by MG132 presents a physiologically-relevant model inasmuch as the proteasome system was compromised but not completely disrupted. Inhibition of UPS by MG132 resulted in a dose-dependent increase of nNOS protein levels in PC12 cells and consequent increase of NO production. Analysis of data in Fig. 3 suggests that the damaging species is downstream of NO generated by nNOS and likely to be ONOO⁻; this view is supported by the increase in total NO₂⁻ level in the presence of nitrate reductase. The cellular generation of ONOO⁻, requires a simultaneous production of O₂⁻ by diverse sources: mitochondria are major sources of O₂⁻ and, therefore, likely sites for ONOO⁻ production. An additional source of O₂⁻ can result from the uncoupling of undegraded nNOS [36,37], which may be favored by cAMP (present in the medium to help differentiate PC12 cells into dopaminergic neurons) [38] by a mechanism entailing activation of protein kinase A and the universal transcription factor cAMP-response element binding protein (CREB). cAMP decreases NO levels by up-regulating the uncoupling of nNOS into O₂⁻ production and, thereby, ONOO⁻ generation [39]. Neither the inducible (iNOS) nor the constitutive (eNOS) isoforms of NOS were detected in this experimental model, thereby making them unlikely sources of the increased ONOO⁻ formation in this model of proteasome inhibition, thus tallying previous reports that proteasome inhibition blocks iNOS induction by preventing activation of NF-κB [40,41].

It may be surmised that generation of ONOO⁻ appears to be a major consequence of a compromised proteasome function and either elevated levels of uncoupled nNOS activity and/or diffusion of NO to mitochondria. Thus, it is reasonable to assume that an enhanced ONOO⁻ formation may contribute partly to neuronal loss upon activation of apoptotic pathways. The nNOS inhibitor only partially protected cells against cell death, thus indicating a complex network of events in the apoptotic sequence induced upon proteasome inhibition and independent of NO: (a) accumulation and stabilization of pro-apoptotic factors such as tBid and Bax [42,43] and (b) induction of ER stress caused by build-up of misfolded proteins that triggers mitochondrion-driven apoptosis [44]. The increased nNOS protein levels following inhibition of proteasome activity correlated with an activation (phosphorylation) of c-Jun N-terminal kinase (JNK); JNK activation was suppressed upon nNOS inhibition, thus

establishing a pecking order in which nNOS upregulation and formation of reactive oxygen species are upstream of JNK phosphorylation. It has been reported that ONOO⁻ can induce JNK activation by (a) direct activation of the mixed lineage kinase (MLK) and leading to mitochondrial cytochrome *c* release [45] and (b) oxidation of Fas (independent of ligands to Fas) and entailing mitochondrial depolarization [30]. The mitochondrion-dependent apoptosis entailed in the above pathways can be explained by translocation of active JNK to the mitochondrial outer membrane [31,46] and/or (ii) recruitment of Bax to mitochondria [47]. Activation of JNK caused, in turn, phosphorylation (inhibition) of the antiapoptotic Bcl-2 protein, Bcl-x_L, a constitutive component of the mitochondrial outer membrane. Predictably, this led to release of mitochondrial cytochrome *c* and activation of caspase-9 and -3. Therefore, mitochondrion-driven apoptosis appears to be a hallmark of nNOS upregulation upon proteasome inhibition. The physiological significance of these findings is supported by increased levels of 3-nitrotyrosine –a biomarker of nitrative stress– in several neurodegenerative diseases [48–50].

A role for the mitochondrial form of NOS (mtNOS) cannot be established in this dopaminergic cell model though the significance of mitochondrial dysfunction in neurodegenerative disorders is of outmost importance. In this regard, mitochondrial damage by [•]NO is potentiated by dopamine with underlying increase in mitochondrial oxidant generation [51]. The contribution of mtNOS to the age-associated increase of [•]NO production is unknown; however, a role for mtNOS gains physiological significance when considering that (a) nNOS is held responsible for elevated [•]NO production during normal aging, (b) mtNOS is the neuronal form of NOS, and (c) mitochondria produce over 30% of the cellular [•]NO [52,53].

Taken together, dopaminergic cells with a partially compromised UPS activity appear to be a suitable model for age-related neurodegenerative disorders, especially Parkinson's disease, and help establish redox-sensitive pathways leading to apoptotic cell death. This model entails an enhanced cellular level of oxidants (be it oxygen- or nitrogen-derived species) that can set up a further inhibition of the proteasome system through a positive feedback mechanism.

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Fig. 1. Time- and dose-dependent inhibition of proteasome-ubiquitin degradation system by MG132 and nNOS protein levels

PC12 cells in differentiation medium were treated with different concentrations of MG132 (0- to 2.5 μM) for 24- or 48 h. Total proteins were separated on 10% SDS PAGE gel. nNOS protein content was detected by western blot using α -nNOS antibody. Dose- and time-dependent increase in nNOS protein level was observed with increase of MG132 concentration. Assay conditions as described in the Materials and methods section.

Fig. 2. Dose-dependent inhibition of UPS and cell viability and cytotoxicity

PC12 cells in differentiation medium were treated with different concentration of MG132 (0- to 10 μ M) for 24 h: (A) Cell viability in the absence (dark bars) and presence (light bars) of 3 mM L-NAME. (B) Cytotoxicity levels in the absence (dark bars) and presence (light bars) of 3 mM L-NAME. (C) Effect of different concentrations of MG132 on cell viability and extent of proteasome activity inhibition. Proteasome inhibition (-●-) was positively correlated to the decrease in cell viability (-○-). Assay conditions as described in the Materials and methods section.

Fig. 3. $\cdot\text{NO}$ formation in PC12 cells: Effect UPS- and nNOS inhibitors

Differentiated PC12 cells were treated with 0.5 μM MG132 in HEPES buffer containing NGF and cAMP. Levels of $\cdot\text{NO}$ were measured by DAN assay as described in the Materials and methods section. (A) Nitrite levels remained unchanged after MG132 treatment. (B) Total nitrite levels increased after MG132 treatment and decreased in the presence of 7-NI.

Fig. 4. Proteasome inhibition and activation of JNK

PC12 cells in differentiation medium were treated with 0.5 μ M MG132 for 24 h (A) Western blots of active (phosphorylated) JNK: effects of inhibitors of proteasome activity, nNOS activity, and JNK activity. Lower bands: western blots of phosphorylated Bcl-x_L under the same conditions as above. (B) Cell viability was assayed as in the conditions described above. Other conditions as in the Materials and methods section.

Fig. 5. Activation of caspase-9 and -3 by MG132 in PC12 cells: Regulation by NOS inhibitors
 PC12 cells were treated with increasing doses of MG132 (0- to 2.5 μM) for 24 h. (A) Western blot of active caspase-9; (B) effect of L-NAME on MG132-dependent activation of caspase-9. (C) Caspase-3 activity modulation by MG132, NOS inhibitors. Assay conditions as described in the Materials and methods section.