Proteasome inhibitor-induced apoptosis is mediated by positive feedback amplification of PKCδ proteolytic activation and mitochondrial translocation

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Abstract

Emerging evidence implicates impaired protein degradation by the ubiquitin proteasome system (UPS) in Parkinson’s disease; however, cellular mechanisms underlying dopaminergic degeneration during proteasomal dysfunction are yet to be characterized. In the present study, we identified that the novel PKC isoform PKCδ plays a central role in mediating apoptotic cell death following UPS dysfunction in dopaminergic neuronal cells. Inhibition of proteasome function by MG-132 in dopaminergic neuronal cell model (N27 cells) rapidly depolarized mitochondria independent of ROS generation to activate the apoptotic cascade involving cytochrome c release, and caspase-9 and caspase-3 activation. PKCδ was a key downstream effector of caspase-3 because the kinase was proteolytically cleaved by caspase-3 following exposure to proteasome inhibitors MG-132 or lactacystin, resulting in a persistent increase in the kinase activity. Notably, MG-132 treatment resulted in translocation of proteolytically cleaved PKCδ fragments to mitochondria in a time-dependent fashion, and the PKCδ inhibition effectively blocked the activation of caspase-9 and caspase-3, indicating that the accumulation of the PKCδ catalytic fragment in the mitochondrial fraction possibly amplifies mitochondria-mediated apoptosis. Overexpression of the kinase active catalytic fragment of PKCδ (PKCδ-CF) but not the regulatory fragment (RF), or mitochondria-targeted expression of PKCδ-CF triggers caspase-3 activation and apoptosis. Furthermore, inhibition of PKCδ proteolytic cleavage by a caspase-3 cleavage-resistant mutant (PKCδ-CRM) or suppression of PKCδ expression by siRNA significantly attenuated MG-132-induced caspase-9 and -3 activation and DNA fragmentation. Collectively, these results demonstrate that proteolytically activated PKCδ has a significant feedback regulatory role in amplification of the mitochondria-mediated apoptotic cascade during proteasome dysfunction in dopaminergic neuronal cells.

Keywords: Ubiquitin Proteasomal System (UPS) • Protein Kinase C delta • Parkinsons disease • Neurodegeneration • Apoptosis • mitochondria

Introduction

Ubiquitin-proteasome system (UPS) is one of the major intracellular proteolysis systems responsible for degradation of damaged or misfolded proteins and proteins involved in various cellular processes including apoptosis. Polyubiquitination of target proteins, which is essential for their recognition and degradation by the 26S proteasome complex, involves a cascade of ubiquitinating enzymes including ubiquitin activating enzyme, ubiquitin conjugating enzyme, and ubiquitin ligase [1]. Parkinson’s disease (PD) is the most common neurodegenerative movement disorder, affecting over 4 million people worldwide, and is becoming more prevalent each year. The disease is characterized by the selective and progressive loss of nigral dopaminergic neurons, with the underlying neuronal death remaining elusive [2]. Lines of evidence for pathogenic roles of dysfunctional UPS in PD include reduced proteasomal activities, selective loss of proteasome subunits in substantia nigra of patients with sporadic PD, and mutation of several genes involved in the UPS degradation pathway in familial PD [2–4]. Accumulation of ubiquitinated proteins in Lewy bodies, presumably due to failure of the clearance of target proteins by
UPS, is indicative of impaired UPS function in PD. Exposure to pharmacological inhibitors of the proteasome replicates some biochemical and pathological characteristics of PD cell culture and animal models. Proteasome inhibition has been previously shown to result in α-synuclein protein aggregation and cell death in various cell models including mesencephalic dopaminergic neurons [2]. The Parkinsonian toxin MPTP has been shown to cause UPS dysfunction and protein aggregation in the substantia nigra [5, 6]. Additionally, other neurotoxic pesticides linked to PD such as rotenone and dieldrin cause proteasome inhibition and protein aggregation [2]. Systemically administered proteasome inhibitors produce inconsistent results in producing Parkinsonian-like pathology in rodents [7–12]. Recently, we and others demonstrated that microinjection of proteasome inhibitors into substantia nigra or striatum effectively reproduces a nigrostriatal dopamine degeneration [13–15]. Despite extensive observations of defective UPS degradation in PD pathogenesis, the cellular and molecular mechanisms leading to dopamine neuronal death following proteasomal dysfunction remain to be characterized. In the present study, we report for the first time that proteolytic activation and mitochondrial translocation of PKCδ play a critical role in apoptotic cell death during proteasome dysfunction in dopaminergic neuronal cells.

Materials and methods

Cell culture and treatment paradigm

The immortalized rat mesencephalic dopaminergic cell line (N27 cells) was grown in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C [16, 17]. Cells were treated with different concentrations of MG-132 or lactacystin dissolved in dimethyl sulfoxide (0.1% DMSO final concentration) for the indicated duration in the experiments. Control groups were treated with 0.1% DMSO.

Mitochondria depolarization assay

The cationic lipophilic fluorescent dye JC-1 accumulates in the matrix of healthy mitochondria through a membrane potential-dependent manner, and thus fluoresces red. However, JC-1 cannot accumulate in mitochondria with collapsed membrane potential, and thus exists in cytoplasm at low concentration as a monomer, which fluoresces green. The intensity of red and green fluorescence provides a reliable measurement of mitochondrial membrane potential. N27 cells grown in 6-well plates were treated with MG-132 prior to incubation with JC-1 dye (Invitrogen, Carlsbad, CA) for 20 min at a final concentration of 2 μg/ml. Red and green fluorescence were determined for the treated cells performed with a flow cytometer with a setting of ‘double-bandpass’ filter Ex/Em 485/535 nm for green fluorescence and Ex/Em 590/610 nm for red fluorescence, and the ratio between red/green was used as an indicator of mitochondria potential.

ROS assay

Flow cytometric analysis of reactive oxygen species in N27 cells was performed with dihydroethidium, as described previously [18–21]. In cytosol, blue fluorescent dihydroethidium can be dehydrogenated by superoxide (O2·−) to form ethidium bromide, which subsequently produces a bright red fluorescence (620 nm). N27 cells were collected by trypsinization and resuspended in Earle’s balanced salt solution (EBSS) with 2 mM calcium at a density of 1.0 × 106 cells/ml. The cell suspension was then incubated with 10 μM hydroethidium at 37°C in the dark. Following addition of MG-132, ROS generation in N27 cells was measured at 0, 20, 40 and 60 min in a flow cytometer (Em/Ex 488/585 nm with 42-nm bandpass). Treatment with H2O2 was used as positive control. ROS levels were normalized as percentage of time-matched control. ROS generation was also examined by monitoring fluorescence using microscopic analysis.

Microscopic assays of ROS production were conducted using dihydroethidium or CM-H2DCFDA by following the protocols suggested by the manufacturers. Briefly, 24 hrs after cells were in culture, N27 cells in 96-well plate were then treated with MG-132 (1, 3 or 5 μM) for 1, 3 or 6 hrs. Incubation of the treated cells with 5 μM dihydroethidium or 10 μM CM-H2DCFDA for an additional 15 min was performed, and the cells were then visualized under fluorescence microscopy with Em at 620 nm and 535 nm for dihydroethidium or CM-H2DCFDA, respectively.

Caspase enzymatic activity assay

Caspase activities were assessed as described in our publications [17, 18]. Cells were lysed with 10 μg digitonin in Tris buffer (50 mM Tris-HCl, 1 mM EDTA, 10 mM EGTA). The supernatants (14,000 g for 40 min) of the lysates were incubated with 50 μM of the fluorogenic substrates Ac-DEVD-AFC, Ac-EDVD-AFC and Ac-LEHD-AFC (Biomol International, Plymouth Meeting, PA) for determination of caspase-3, -8 and -9 activities, respectively. Levels of cleaved substrate (active caspase) were monitored performed with a fluorescence plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA, Ex/Em 460/530 nm). Protein concentration was determined by the Bradford method and used for normalization of caspase activity.

Subcellular fractionation, preparation of cell lysate and Western blot

Mitochondria isolation was conducted, as described previously [22] with modification. Cells were resuspended in homogenization buffer (pH 7.5, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM DTT, 0.1 mM PMSF and protease inhibitors), and homogenized with a glass Dounce homogenizer. Unlysed cells, cell debris and nuclei were removed by centrifugation at 16,000 g for 40 min For Western blot, samples were resolved on SDS-PAGE and then transferred to nitrocellulose membranes for immunoblotting with antibodies recognizing PKCα (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, 1:2000), V5 (Invitrogen, 1:5000),...
cytochrome c (BD Pharmingen, San Jose, CA, 1:1500). Smac (ProSci, Poway, CA 1:500) or COX IV (Invitrogen, 1:1500).

**In vitro mitochondria release assay**

Isolated mitochondria were resuspended in the same isolation buffer at a concentration of 2.0 mg/ml. For the release assay [22], 40 μl mitochondria suspension was incubated with MG-132 at 30°C for 60 min Triton X-100 (0.2%, v/v) was included as positive control to release cytochrome c. After incubation, mitochondria were spun down and the supernatant was collected for the SDS-PAGE and immunoblotted for cytochrome c (BD Pharmingen, San Jose, CA, 1:1500).

**PKCs kinase assay**

The enzymatic activity of PKCs was measured with an immunoprecipitation kinase assay, as described previously [23]. Cells were lysed with lysis buffer (25-mM HEPES pH 7.5, 20-mM NaCl, 1.5-mM MgCl2, 0.2-mM EDTA, 0.5-mM DTT, 10-mM NaF, 4 μg/ml aprotinin, and 4 μg/ml leupeptin). The cell lysate was centrifuged at 10,000 g for 20 min to obtain the supernatant as cytosolic fraction. Cytosolic protein (500 μg) was immunoprecipitated with 2 μg PKCs antibody. The immunoprecipitates were washed 3 times with 2X kinase buffer (40 mM Tris pH 7.4, 20 mM MgCl2, 20 μM ATP, and 2.5 mM CaCl2), and resuspended in 20 μl of the same buffer. The reaction was initiated by adding 20 μl of reaction buffer (0.4 mg Histone H1, 50 μl phosphatidylserine, 4.1 μM dioleyl-glycerol, and 5 μCi of [γ-32P] ATP) to the resuspended immunoprecipitates. After 10-min incubation, samples were separated on 12% SDS-PAGE. The radioactively labelled histone H1 was detected performed with a Phosphorimage system (Personal Molecular Imager, FX model, Bio-Rad Labs, Hercules, CA, USA) and analysed with Quantity One 4.2.0 software.

**Plasmid construction and siRNA synthesis**

Full-length wild-type (wt) PKCs-GFP and PKCs<sup>D327A</sup>-GFP in pEGFP-N1 vector were obtained from Dr. Mary Reayland (University of Colorado, Boulder, CO). Full-length (PKCs-FL), the regulatory fragment (PKCs-RF) and the catalytic fragment (PKCS-CF) of PKCs were amplified from wt-PKCs-GFP in the pEGFP-N1 vector, and PKCs<sup>D327A</sup> (caspase-3 cleavage-resistant mutant, PKCs-CRM) was amplified from PKCs<sup>D327A</sup>-GFP in pEGFP-N1 vector by PCR. The PCR product was then cloned into the p lent6/VS-D-TOPO expression vector by following the procedure provided by the manufacturer (Invitrogen, CA). The primers used were: 5’-CACCATTGCACCCTTCTCTGCTC3’ (forward primer for PKCs-FL, PKCs-CRM and PKCs-RF) and 5’-AATGTCGAAATTGCTCAAAC-3’ (reverse primer for PKCs-FL, PKCs-CRM and PKCs-CF), 5’-ACTTCGAGACTTTCTTCGTT-3’ (reverse primer for PKCs-RF) and 5’-CACCATTGCCAACCAGCGACTTGGTGCAA-3’ (forward primer for PKCs-CF). To achieve mitochondria-targeted expression, PKCs-RF and PKCs-CF were cloned into the pCMV/Myc/Mito vector (Invitrogen) at Sal I and Not I sites by following standard cloning procedure. LacZ was cloned into the same vector to serve as a control. The primers used included: 5’-ATATGCTGGTCG-3’ (reverse primer for PKCs-CF), 5’-ATATATACGCGCACATATAGCGCGCAGGCGCAATGCGCGACAGA-3’ (forward primer for PKCs-CF), 5’-ATATAGCGCGGCGAATTGCGGCTACAAAC3’ (reverse primer for PKCs-FL and PKCs-CF) and 5’-ATATATGGCGCGCAGCTCAGACTCAGGACAGCAGTCGTGG-3’ (reverse primer for PKCs-RF).

Synthesis of siRNA duplex specifically targeting PKCs and a non-specific siRNA was conducted as described in our previous publications [16, 24]. Chemically synthesized sense and antisense transcription templates contain a leader sequence complementary to the T7 promoter primer and an encoding sequence for siRNA. Following the annealing of the transcription templates with T7 promoter primer, hybridized DNA oligonucleotides were extended using Klenow DNA polymerase to form double-stranded transcription templates. An *in vitro* transcription reaction was then conducted with antisense or sense templates using T7 RNA polymerase, and the resulting RNA transcripts were annealed to form siRNA duplexes before removal of the leader sequence with single-strand-specific ribonuclease.

**Cell transfection**

The expression vectors (pLenti-PKC<i>α</i>-CRM and pLenti-LacZ) were cotransfected with packaging plasmids provided by the manufacturer into 293 FT cells performed with Lipotectamine<sup>TM</sup> 2000 reagent for virus production (Invitrogen). The lentiviruses derived from the transfected 293 FT cells were used for transfection of pLenti-PKCs-α-CRM and pLenti-LacZ in N27 cells. For stable expression, cells were selected with blasticidin (10.0 μg/ml). Transient transfection was conducted performed with either AMAXA Nucleofector reagent (Amx Inc., Gaithersburg, MD) or the jetPEI<sup>TM</sup> DNA in vitro transfection reagent (Polyplus-transfection Inc., New York, NY). For AMAXA electroporation, approximately 2 × 10<sup>6</sup> cells were suspended in 100 μl prepared Nucleofector<sup>TM</sup> solution V, and then mixed well with expression vectors (PKCs-α-CF or PKCs-RF), or siRNA (non-specific (NS) siRNA or PKCs siRNA) before electroporation. Based on our previous studies [16], 25 nM siRNA-PKCs and siRNA-NS were used for transfection. This concentration effectively suppresses PKCs in N27 cells [16, 24]. Transfection efficiency was determined by pmaxGFP transfection, which was used as a control group for the caspase-3 assay. For transfection of mitochondria-targeted vectors, plasmids (2.0 μg) were first mixed with 100 μl sterile sodium chloride (150 mM) to make the plasmid solution. The jetPEI<sup>TM</sup> solution was made by mixing 4.0 μl jetPEI<sup>TM</sup> reagent with 100 μl sterile sodium chloride. The jetPEI<sup>TM</sup> solution was then added to the plasmid solution and mixed well, and 200 μl jetPEI<sup>TM</sup>/DNA mixture was incubated at room temperature for 25 min before being added into culture wells. Transfected cells were viewed with confocal microscopy.

**DNA fragmentation assay**

DNA fragmentation was measured performed with a Cell Death Detection ELISA Plus Assay Kit (Roche Applied Science, Indianapolis, IN) as previously described [18]. Briefly, cells were resuspended with the lysis buffer provided in the assay kit. The lysate was centrifuged at 200 × g, and 20 μl of supernatant was incubated for 2 hrs with the mixture of HRP-conjugated antibody cocktail that recognizes histones, and single- and double-stranded DNA. After washing away the unbound components, the final reaction product was measured colorimetrically, with ABTS as an HRP substrate performed with a spectrophotometer at 405 nm (490 nm as reference).

**Immunocytochemistry and TUNEL staining**

Immunofluorescence was conducted as described previously [25]. Briefly, 24 hrs after plasmid transfection, N27 cells cultured on
coverslips pre-coated with poly-L-lysine were washed with PBS, and fixed with 4% paraformaldehyde. After permeabilization with 0.2% Triton X-100, cells were incubated with blocking buffer (5% BSA, 5% goat serum in PBS) to minimize non-specific binding. For double staining, cells were incubated overnight with antibodies recognizing Myc tag (Abcam, Mouse monoclonal Ab 1:200) and cleaved caspase-3 (Cell signalling, Rabbit monoclonal Ab, 1:100). Then Myc tagged fusion proteins and cleaved caspase-3 were visualized with Cy3 conjugated anti-mouse and Alexa 488-conjugated anti-rabbit secondary antibodies, respectively. The images were analysed performed with Nikon C1 confocal microscopy.

**Results**

### Proteasome inhibition by MG-132 precedes mitochondria depolarization

We used the proteasome inhibitor MG-132 to induce ubiquitin proteasome dysfunction in N27 dopaminergic cells. To systematically examine the effect of the proteasome inhibitor MG-132 on dopaminergic cells, we first performed a detailed time course analysis of chymotrypsin-like proteasomal activity. As shown in Fig. 1A, MG-132 exposure led to a rapid inhibition of proteasomal activity, with more than 70% inhibition within 5 min ($P < 0.001$).

Next, we examined whether MG-132 has any effect on mitochondrial membrane potential and ROS generation. Determination of membrane potential by JC-1 showed a gradual and steady reduction starting at 30 min, with a 50% decrease in mitochondrial membrane potential over 120 min of MG-132 ($P < 0.001$). On the contrary, no significant elevation of ROS was noted during MG-132 treatment as measured by flow cytometry. To further confirm ROS production over prolonged exposure to MG-132, fluorescence microscopic analysis were performed. N27 cells were incubated with two different ROS sensitive dyes, dihydroethidine and CM-H$_2$DCFDA, prior to treatment with MG-132 (0.1, 1, 3 or 5 µM) and ROS production was monitored over 6 hrs. As shown
in Fig. 1D, no significant change in ROS production was detected with either dye in N27 cells exposed to 5.0 μM MG-132 for 6 hrs. Additionally, lower doses (0.1-3.0 μM) did not generate any ROS (data not shown). However, we observed a significant ROS production in the 50 μM H2O2 treatment (positive control; Fig. 1D). These data indicate that proteasomal inhibition, which precedes the dissipation of mitochondria membrane potential, is independent of oxidative insult.

Proteasome inhibition by MG-132 triggers mitochondria-mediated apoptosis

Since MG-132 produced depolarization of mitochondria, we examined whether the mitochondria-dependent apoptotic pathway is activated during proteasome inhibitor treatment in dopaminergic cells. First, we examined the effect of MG-132 on the release of proapoptotic molecules from mitochondria to cytosol. As shown in Fig. 2A, cytosolic fractions from MG-132 treated N27 cells showed a time-dependent release of cytochrome c and Smac into cytosol in a similar temporal pattern (Fig. 2A). No detection of the mitochondrial inner membrane protein COX IV in the cytosolic fraction indicated that the cytosolic fraction was free of mitochondrial contamination. To determine whether MG-132-induced release of proapoptotic factor results from a direct effect of the proteasome inhibitor on mitochondria, we measured cytochrome c release in isolated mitochondria following MG-132 treatment. The results showed no significant increase in cytochrome c release from isolated mitochondria (Fig. 2B), indicating that mitochondrial release of cytochrome c occurred in cells as a consequence of proteasome inhibition by MG-132, but not due to the direct stimulatory effect of MG-132 on mitochondria.

Formation of the apoptosome complex by mitochondria-released cytochrome c, Apaf-1, and dATP/ATP is essential for the activation of initiator caspase-9, which then activates downstream effector caspase-3. As shown in Fig. 2C, caspase-9 activity significantly increased 30 min after MG-132 treatment (P < 0.05), and then dramatically elevated three- to fourfold at 90 and 180 min (P < 0.001). We also measured caspase-8 activity to determine whether a non-mitochondria-mediated apoptotic pathway contributes to cell death during proteasome inhibitor treatment. The results showed only a minimal activation of caspase-8 activity only after the 120-min treatment of MG-132 (Fig. 2C). Next, activity of the key effector caspase commonly known as caspase-3 was measured. MG-132 treatment resulted in a time-dependent increase starting at 90 min, and the activity was dramatically increased at 120 and 180 min (P < 0.001) (Fig. 2D). Notably, caspase-3 activation was completely blocked by the caspase-9 inhibitor LEHD-fmk (Fig. 2E), indicating that caspase-9 is the major upstream caspase responsible for MG-132-induced caspase-3 activation.
Proteasomal inhibition leads to proteolytic activation of PKCβ

Recently, we demonstrated that PKCβ is highly expressed in mouse nigral dopamine neurons [24], and that kinase can be proteolytically activated by caspase-3 during oxidative stress-induced dopaminergic degeneration [16, 18, 21, 23, 26]. Since we found a dramatic increase in caspase-3 activation during MG-132 treatment, we examined whether PKCβ is proteolytically activated in N27 cells. Western blot analysis revealed dose-dependent and time-dependent proteolytic cleavage of PKCβ following exposure to the 1-5 μM MG-132 for 1, 3 or 6 hrs (Fig. 3A and C). The proteolytic cleavage was caspase-3-dependent, since it was diminished markedly by the caspase-3 inhibitor zDEVD-fmk (50 μM), as well as by the pan-caspase inhibitor zVAD-fmk (100 μM) (Fig. 3A), indicating that the proteasome inhibitor induces caspase-3-dependent proteolytic cleavage of PKCβ. To assess the effect of proteolytic cleavage of PKCβ on its kinase activity, the PKCβ was immunoprecipitated from cell lysates and an immunokinase assay was performed using [γ-32P]-ATP and histone H1 substrate. Analysis of the intensity of radioactively labelled histone H1 bands indicated that MG-132 exposure results in a 282% increase in kinase activity of PKCβ (Fig. 3B). Inhibition of PKCβ proteolytic cleavage either by caspase-3 inhibitors zDEVD-fmk (50 μM) or rottlerin (2.5 μM), or the pan-caspase inhibitor zVAD-fmk (100 μM) diminished its kinase activity, indicating that caspase-3 mediates PKCβ proteolytic cleavage and significantly activates its kinase activity (Fig. 3B). In order to verify that proteasome inhibition triggers proteolytic activation of PKCβ, we used another highly specific and irreversible proteasome inhibitor, lactacystin. Treatment with lactacystin (5.0 μM) for 90 or 120 min also caused a marked increase in the proteolytic cleavage of PKCβ (Fig. 3D), substantiating the proteolytic activation of PKCβ as a result of proteasomal inhibition in dopaminergic neuronal cells.

Activated PKCβ as mediator for MG-132-induced mitochondria-mediated apoptosis

Next, we examined the role of proteolytically cleaved PKCβ in apoptosis by using PKCβ catalytic fragment (PKCβ-CF) and PKCβ regulatory fragment (PKCβ-RF). N27 cells were transfected with PKCβ-CF or PKCβ-RF, and the transfection efficiency was estimated by the cotransfected GFP plasmids (Fig. 4A). Measurement of caspase-3 activity revealed a significant increase in the caspase-3 activity in PKCβ-CF-transfected cells as compared to RF-transfected or GFP-transfected cells, suggesting that kinase active PKCβ-CF is responsible for its proapoptotic effect in dopaminergic cells (Fig. 4B). Additionally, pre-treatment with the PKCβ-specific inhibitor rottlerin also significantly attenuated MG-132-induced caspase-9 and -3 activation (Fig. 4C and D), indicating that PKCβ activation indeed contributes to caspase activation following exposure to the proteasome inhibitor MG-132. However, MnTBAP, a superoxide dismutase (SOD) mimic, failed to attenuate caspase-3 activation following MG-132 exposure (Fig. 4E). Further, we and others have recently demonstrated a positive feedback activation of caspase-3 and caspase-9 by proteolytically activated PKCβ during apoptotic cell death [21, 27]. Thus, a positive feedback loop would in part explain the observed inhibition of caspase-9 activation by rottlerin (Fig. 4C). This finding suggests that the proapoptotic effect of PKCβ proceeds through the mitochondria-mediated apoptosis pathway.

Mitochondrial translocation of active PKCβ induces apoptosis

Next, we examined whether activated PKCβ translocates to subcellular organelles to promote its proapoptotic function. As shown in Fig. 5A, MG-132 treatment resulted in substantial and time-dependent accumulation of cleaved PKCβ in the mitochondrial fraction, and only a slight elevation of full-length PKCβ was observed. COX IV protein was used as marker of mitochondrial protein in the Western blot analysis (Fig. 5A). Together, these results indicate proteasomal inhibition proteolytically activates PKCβ and causes translocation to mitochondria.

To understand whether mitochondrial translocation of PKCβ-CF is functionally related to its proapoptotic effect, mitochondria-targeted expression of PKCβ-CF and PKCβ-RF was achieved performed with pCMV/myc/mito vector. Double immunostaining for myc tag (red) and active caspase-3 (green) revealed activation of caspase-3 in the PKCβ-CF-transfected cells, but not in the PKC-RF or LacZ transfected cells (Fig. 5B). Also, cells transfected with PKCβ-CF, but not PKCβ-RF or LacZ, were TUNEL positive (Fig. 5C). Collectively, these results demonstrate that mitochondrial translocation of PKCβ-CF can amplify the apoptotic cascade during proteasomal dysfunction.

Suppression of PKCβ proteolytic activation protects cells from mitochondria-mediated apoptosis following proteasome inhibitor exposure

To further substantiate that proteolytic activation is primarily responsible for the feedback amplification of mitochondria-mediated apoptotic signalling during dopaminergic apoptosis, a caspase-3 cleavage-resistant mutant of PKCβ (PKCβΔD327A, PKCβ-CRM) was introduced into N27 cells using a lentivirus expression system and cells stably expressing PKCβ-CRM were generated. PKCβ-CRM expressing cells are more resistant to MG-132-induced PKCβ proteolytic cleavage (Fig. 6A). Furthermore, the PKCβ-CRM cells were more resistant to MG-132-induced mitochondria-mediated apoptosis, as demonstrated by the significant reduction of caspase-9, caspase-3 activation, and DNA fragmentation compared to LacZ-transfected cells (Fig. 6B–D). Together, these results demonstrate proteolytic activation of PKCβ promotes apoptotic cell death via a feedback amplification of the mitochondria-mediated caspase cascade.
Inhibition of mitochondria-mediated apoptosis by PKCδ siRNA

To further confirm the critical role of PKCδ in mitochondria-mediated apoptosis during proteasome inhibition, we used a RNAi approach. siRNA duplex specifically targeting PKCδ [16, 24] was introduced into N27 cells using electroporation transfection, and then caspase-9, caspase-3, and DNA fragmentation were assayed. The results revealed a remarkable inhibitory effect of siRNA-PKCδ on the activation of caspase-9 (Fig. 7A), caspase-3 (Fig. 7B) and DNA fragmentation (Fig. 7C). Non-specific siRNA treatment did not alter these apoptotic markers. Again, a positive feedback loop would in part explain the observed inhibition of caspase-9 activation by PKCδ siRNA (Fig. 7A).

Collectively, these results clearly confirm that PKCδ regulates the mitochondria-mediated apoptotic cascade following proteasomal dysfunction in dopaminergic neuronal cells.

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Discussion

The present study reveals an important regulatory role of PKC in mitochondria-mediated apoptosis in mesencephalic dopaminergic neuronal cells following proteasome inhibition. We demonstrated activation of the mitochondria-mediated apoptosis cascade and proteolytic activation of PKC during proteasome inhibition. Importantly, we found that proteolytic activation and mitochondrial translocation of PKC underlie its positive feedback amplification of mitochondria-mediated apoptosis during proteasome dysfunction in mesencephalic dopaminergic neuronal cells. This mitochondria-dependent proapoptotic capacity of PKC sheds light on degenerative processes of dopaminergic neurons mediated by UPS dysfunction in PD.

In addition to mitochondria dysfunction and oxidative stress, UPS dysfunction has recently been recognized as a key pathophysiological process of PD. Previous studies have revealed that the substantia nigra particularly suffers from UPS dysfunction in the brains of patients with sporadic PD [28, 29]. Mutations in genes of the ubiquitin ligase Parkin and the deubiquitin enzyme UchL-1 in familial PD have provided further evidence for the contributory roles of impaired UPS function in PD [2, 30, 31]. Notably, proteasome inhibitors have been shown to reproduce some key features of PD, including neuronal death [32–34]. However, underlying cell death mechanisms during UPS dysfunction remain to be determined. Recently, we and other have shown that dopaminergic neurons in mesencephalic culture are more susceptible to proteasomal inhibition than non-dopaminergic cells [15, 28, 32, 33, 35].
Fig. 5 Effect of mitochondria-localized active PKCα on apoptosis. (A) Mitochondrial translocation of proteolytically activated PKCα. Mitochondrial fraction was prepared from cells exposed to 5.0 μM MG-132 for 90 or 120 min. Mitochondrial lysates were separated on SDS-PAGE and immunoblotted with PKCα antibody, and the membrane was reprobed with COX IV to show equal protein loading. (B) Mitochondrial-localized active PKCα activates caspase-3. After N27 cells were transfected with pCMV/myc/mito containing coding sequence for LacZ, PKCα-RF or PKCα-CF, double immunostaining was conducted using the mouse Myc tag primary antibody and rabbit active caspase-3 antibody. The Myc tag and active caspase-3 were visualized using confocal microscopy using Cy3 conjugated anti-mouse (red) and Alexa-488 conjugated anti-rabbit (green) secondary antibodies. (C) TUNEL staining in the transfected cells. After transfection for 24 hrs, cells were subjected to TUNEL staining (green) and immunostaining with Myc tag antibody (red). The images were analysed with fluorescence microscopy. The inserts show higher magnification pictures (600×).
Additionally, we have shown dopaminergic neurons in nigral regions are more sensitive than non-dopaminergic cells following intra-nigral injection of MG-132 [15]. In the present study, we showed substantial reduction of proteasomal activity shortly after exposure to a low dose (5.0 \mu M) of MG-132 (70\%, Fig. 1A), which was followed by progressive dissipation of mitochondrial membrane potential (Fig. 1B). Mitochondrial depolarization has been extensively observed during apoptosis, concurrent with mitochondrial release of proapoptotic molecules in apoptosis models, including PD models [36]. Following MG-132 treatment, cytosolic cytochrome c and Smac levels progressively increased in N27 cells (Fig. 2A). It appears that mitochondrial release of cytochrome c occurred as a consequence of proteasome inhibition by MG-132, but not due to a direct stimulatory effect of MG-132 on mitochondria, because incubation of isolated mitochondria with MG-132 failed to trigger mitochondrial release of cytochrome c (Fig. 2B).

Several proteins important for regulation of mitochondria-dependent apoptosis, such as p53, Bax, PUMA, and BAD, have previously been shown to be degraded by the proteasome. Presumably, the inadequate proteasomal degradation of these proteins increases their mitochondrial association or translocation, and thus actively contributes to the release of cytochrome c and resulting mitochondrial functional impairment [37]. However, we cannot rule out the role of other cellular mechanisms such as lipoxigenases, ER stress and autophagy in proteasome inhibitors-induced cell death processes. Recently, lipoxigenases have been shown to play a key role in organelle degradation and mitochondria-dependent neuronal cell death [38, 39]. It has also been shown that accumulation of ubiquitinated proteins can facilitate the formation of macroautophagy, a process in which impaired organelles including mitochondria, are targeted for degradation by lysosome [40]. Presently, it is unclear whether proteasome inhibition in neuronal cells can

Fig. 6 Suppression of PKC\(\delta\) proteolytic activation protects cells from mitochondria-mediated apoptosis during proteasome inhibition. (A) Suppression of PKC\(\delta\) proteolytic cleavage by PKC\(\delta\)-CRM. N27 cells stably transfected with LacZ (as control) and PKC\(\delta\)-CRM were treated with 5.0 \mu M MG-132. Equal amounts of protein from the LacZ and CRM cells were separated on SDS-PAGE and transferred onto nitrocellulose membrane for immunoblotting with PKC\(\delta\) antibodies. The membrane was reprobed and blotted with \(\beta\)-actin antibody. (B, C and D) Suppression of mitochondria-mediated apoptosis by PKC\(\delta\)-CRM. Caspase-9 activity (B), caspase-3 activity (C) and DNA fragmentation (D) were determined for LacZ and CRM cells exposed to MG-132 for 120 min. The values are expressed as the percentage of control cells. Results represent mean \(\pm\) S.E.M from 6–8 samples. \(* P < 0.05\) and \(**\ P < 0.001\).
trigger autophagy in dopaminergic neurons. Future studies will clarify the integrative role of apoptosis, autophagy and programmed necrosis in the neurodegenerative process following proteasomal dysfunction. To our knowledge, this is the first report demonstrating that proteasome inhibition activates mitochondria-mediated apoptotic signalling in dopaminergic cells.

Association of cytosolic cytochrome c with Apaf-1 and dATP/ATP as the apoptosome complex is essential for the activation of initiator and effector caspases. Following MG-132 treatment, significant activation of caspase-9 and -3 but not caspase-8 was observed for 90 min (Fig. 2C and D). Unexpectedly, caspase-8 and -9 activities were slightly but significantly reduced at early stages (up to 60 min) of MG-132 treatment (Fig. 2C), presumably due to compensatory action of anti-apoptotic proteins such as IAPs or Mcl-1 upon proteasome inhibition [41, 42]. In comparison to caspase-9 and -3 activation, the slight activation of caspase-8 at late time-points (150 and 180 min) agrees with previous reports demonstrating caspase-8 activation resulting from caspase-9 and -3 activation [43]. It appears that caspase-8 plays only a minor role in proteasomal dysfunction in dopaminergic cells. Notably, the apoptotic cell death during MG-132 was predominantly through the mitochondria-mediated apoptotic pathway, since caspase-3 activation was completely suppressed by the caspase-9 inhibitor LEHD-FMK (Fig. 2E), indicating that caspase-9 is the major upstream initiator caspase during proteasomal dysfunction.

Protein kinase Cδ (PKCδ), a member of the novel PKC family, has a structurally and functionally distinct N-terminal regulatory fragment, a C-terminal catalytic fragment, and a medial hinge region [44]. Proteolytic cleavage of PKCδ at the hinge region by caspase-3 represents one of the primary means of its activation, in addition to membrane translocation or phosphorylation [17]. Proteolytic cleavage of PKCδ physically dissociates the auto-inhibitory regulatory fragment from its catalytic fragment, thus permanently activating its kinase activity. Tyr-311 phosphorylation of PKCδ is important for its caspase-3-mediated proteolytic cleavage.
Previously, we showed that proteolytically activated PKC\(_{\alpha}\) plays a key role in mediating oxidative stress-induced apoptosis in dopaminergic neuronal cells [16, 18, 21, 46]. The possible mechanisms of positive feedback activation of caspase-3 in dopaminergic neuronal cells are yet to be identified. Although phosphorylation of caspase-3 by full-length PKC\(_{\alpha}\) has been shown to increase enzymatic activity of caspase-3 in monocytes [47], we did not find caspase-3 phosphorylation by PKC\(_{\alpha}\) in dopaminergic cells (unpublished observations). It appears that PKC\(_{\alpha}\) amplifies caspase-3 activation via distinct mechanisms when it is proteolytically activated in dopaminergic neuronal cells. Proteolytic activation of PKC\(_{\alpha}\) in dopaminergic cells appears to depend on caspase-3 activation in N27 cells exposed to proteasome inhibitors (Fig. 3A–C). The direct proapoptotic effect of PKC\(_{\alpha}\)-CF is manifested by the elevation of caspase-3 activity in PKC\(_{\alpha}\)-CF transfected cells (Fig. 4B), consistent with apoptotic function of PKC\(_{\alpha}\) proteolytic activation. In addition, PKC\(_{\alpha}\) likely enhances activation of caspase-9, which further activates the downstream effector caspase-3, since the PKC\(_{\alpha}\)-specific inhibitor rottlerin attenuates activation of caspase-3 and upstream initiator caspase-9, at least in our study (Fig. 4C and D). Emerging evidence has indicated that rottlerin, a previously well-accepted specific inhibitor for PKC\(_{\alpha}\), actually possesses some biological function other than PKC\(_{\alpha}\) inhibition, such as uncoupling mitochondria [48]. Rottlerin has been shown to be a potent inhibitor of PKC\(_{\alpha}\), with an IC\(_{50}\) of 3-6 \(\mu\)M, whereas the K\(_{i}\) for other PKC isoforms is 5-10 times higher. Other studies have shown rottlerin may inhibit other kinases such as CaM kinase III, PRAK and MAPKAP-K2 as well as other cellular targets [48–52]. Since rottlerin is not a highly specific inhibitor for PKC\(_{\alpha}\), we used multiple approaches including siRNA, dominant-negative mutant and cleavage-resistant mutants in our studies. In the present study, effective suppression of mitochondria-mediated apoptosis either by PKC\(_{\alpha}\) cleavage-resistant mutant or by PKC\(_{\alpha}\) siRNA clearly suggests PKC\(_{\alpha}\) activation truly underlies its pro-apoptotic capacity following MG-132 exposure.

Induction of ROS generation was observed during prolonged exposure to proteasome inhibitors Bortezomb [53], MG-132, lactacystin [54], and PS-341 [55] in several non-neuronal cell lines. Oxidative stress has been demonstrated to activate caspase-3 and PKC\(_{\alpha}\) in N27 cells [18, 23]. In an attempt to determine whether dopaminergic apoptosis following MG-132 exposure involves oxidative stress, ROS generation was measured; no significant increase in ROS generation was noted (Fig. 1C and D). Additionally, the antioxidant MnTBAP, which has been previously shown to effectively inhibit caspase-3 activation during oxidative stress in N27 cells [18], failed to attenuate caspase-3 activation induced by MG-132 (Fig. 4E). Our data suggest that ROS generation plays a negligible role in apoptotic cell death following proteasome inhibition in mesencephalic dopaminergic neuronal cells.

The mitochondria-dependent proapoptotic capacity of active PKC\(_{\alpha}\) is indicated by suppression of caspase-9 activation by rottlerin, was accompanied by mitochondrial translocation of PKC\(_{\alpha}\). This translocation of proteolytically activated PKC\(_{\alpha}\) following proteasome inhibition is consistent with the existing hypothesis that the mitochondrion is a major target organelle that determines the fate of cell survival and death [56]. PKC\(_{\alpha}\) has been shown to accumulate in nuclei [57], golgi [58] and endoplasmic reticulum [59] in various cell types. In an attempt to determine whether mitochondrial translocation of proteolytically activated PKC\(_{\alpha}\) underlies its proapoptotic effect, marked activation of caspase-3 was noted in the N27 cells expressing mitochondria targeted PKC\(_{\alpha}\)-CF (Fig. 5B), but not PKC\(_{\alpha}\)-RF or LacZ. This indicates that mitochondrial translocation of PKC\(_{\alpha}\)-CF possibly underlies its feedback amplification of caspase activation. Considering the multiple pathways that lead to...
PKCs activation, we conducted additional experiments to verify that PKCs proteolytic activation mediates its mitochondria-dependent proapoptotic effect. Expression of a caspase-3 cleavage-resistant mutant of PKCα (PKCα-CRM), which effectively inhibited the proteolytic cleavage of endogenous PKCα (Fig. 6A), significantly attenuated the activation of mitochondria-mediated apoptosis triggered by MG-132 (Fig. 6B–D). These findings are consistent with a recent study showing that PKCα-CRM reduces the mitochondrial release of cytochrome c in UV-challenged keratinocytes [46]. Phosphorylation of mitochondrial resident proteins by active PKCs likely underlies its effect on mitochondria-mediated apoptosis. Several mitochondrial proteins have been characterized as candidate substrates of PKCs, including phospholipid scramblase [60] and pyruvate dehydrogenase kinase [61]. Likely, elevated levels of BAD, due to its incomplete degradation by the proteasome, also participate in the mitochondria-dependent proapoptotic effect of PKCs in dopaminergic cells, given the previous finding that PKCα mitochondrial translocation is accompanied by an increase in the level of BAD following cardiac ischaemia [62]. We also recently reported that systemic administration of PKCα inhibitor alleviates the nigrostriatal dopaminergic degeneration in an MPTP-induced animal model of PD; PKCα inhibitor has been previously shown to suppress dopaminergic apoptosis involving proteolytic activation of PKCs in the N27 cells [18, 24]. Therefore, we believe that PKCα inhibition is expected to exert a similar neuroprotection in an MG-132 in vivo model. We are currently investigating the role of PKCα using RNAi-based gene knockdown and PKCα-knockout animals. Future studies should focus on identifying the key target molecule in mitochondria that contributes to amplification of apoptotic cell death following proteasome dysfunction.

As summarized in Fig. 8, the present study demonstrates that proteolytic activation and mitochondrial translocation of PKCα underlies its feedback activation of mitochondria-mediated apoptosis during proteasome dysfunction in mesencephalic dopaminergic neuronal cells. The high expression of PKCα in nigral dopaminergic neurons and the convergence of proteasomal and mitochondrial dysfunction at the level of PKCα demonstrate that the kinase is a crucial proapoptotic signalling molecule in dopaminergic degenerative processes. This knowledge advances our understanding of the pathogenesis of nigrostriatal degeneration and validates PKCα as potential target for therapeutic intervention of PD.

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