

Protein kinase D1 (PKD1) activation mediates a compensatory protective response during early stages of oxidative stress-induced neuronal degeneration

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Protein kinase D1 (PKD1) activation mediates a compensatory protective response during early stages of oxidative stress-induced neuronal degeneration

Arunkumar Asaithambi, Arthi Kanthasamy, Hariharan Saminathan, Vellareddy Anantharam and Anumantha G Kanthasamy*

Abstract

Background: Oxidative stress is a key pathophysiological mechanism contributing to degenerative processes in many neurodegenerative diseases and therefore, unraveling molecular mechanisms underlying various stages of oxidative neuronal damage is critical to better understanding the diseases and developing new treatment modalities. We previously showed that protein kinase C delta (PKC δ) proteolytic activation during the late stages of oxidative stress is a key proapoptotic signaling mechanism that contributes to oxidative damage in Parkinson's disease (PD) models. The time course studies revealed that PKC δ activation precedes apoptotic cell death and that cells resisted early insults of oxidative damage, suggesting that some intrinsic compensatory response protects neurons from early oxidative insult. Therefore, the purpose of the present study was to characterize protective signaling pathways in dopaminergic neurons during early stages of oxidative stress.

Results: Herein, we identify that protein kinase D1 (PKD1) functions as a key anti-apoptotic kinase to protect neuronal cells against early stages of oxidative stress. Exposure of dopaminergic neuronal cells to H₂O₂ or 6-OHDA induced PKD1 activation loop (PKD1^{S744/748}) phosphorylation long before induction of neuronal cell death. Blockade of PKC δ cleavage, PKC δ knockdown or overexpression of a cleavage-resistant PKC δ mutant effectively attenuated PKD1 activation, indicating that PKC δ proteolytic activation regulates PKD1 phosphorylation. Furthermore, the PKC δ catalytic fragment, but not the regulatory fragment, increased PKD1 activation, confirming PKC δ activity modulates PKD1 activation. We also identified that phosphorylation of S916 at the C-terminal is a preceding event required for PKD1 activation loop phosphorylation. Importantly, negative modulation of PKD1 by the RNAi knockdown or overexpression of PKD1^{S916A} phospho-defective mutants augmented oxidative stress-induced apoptosis, while positive modulation of PKD1 by the overexpression of full length PKD1 or constitutively active PKD1 plasmids attenuated oxidative stress-induced apoptosis, suggesting an anti-apoptotic role for PKD1 during oxidative neuronal injury.

Conclusion: Collectively, our results demonstrate that PKC δ -dependent activation of PKD1 represents a novel intrinsic protective response in counteracting early stage oxidative damage in neuronal cells. Our results suggest that positive modulation of the PKD1-mediated compensatory protective mechanism against oxidative damage in dopaminergic neurons may provide novel neuroprotective strategies for treatment of PD.

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Background

Oxidative stress-induced neuronal damage has been implicated in many neurodegenerative disorders including Parkinson's disease (PD), Alzheimer's diseases, ALS, Huntington's diseases and stroke [1-7]. Neuronal cells maintain an oxidant/antioxidant homeostatic balance, and any breach in redox homeostasis causes excessive ROS production, resulting in oxidative damage [8-10]. Oxidative stress triggers apoptosis through activation of many signaling molecules including kinases and proteases [11-15], and the roles of these signaling molecules in dopaminergic cell death are being actively investigated. Recently, we demonstrated that the proteolytic activation of PKC δ , a novel PKC family member, mediates apoptosis in cell culture and animal models of PD [15-19].

PKC δ can be activated by membrane translocation, phosphorylation, or proteolytic cleavage by caspase-3, leading to persistently active catalytic fragments. We previously showed that various oxidative stressors like H₂O₂, MPP⁺ and 6-OHDA induce PKC δ cleavage to increase the kinase activity and apoptosis in dopaminergic cells [20,15,21,16]. The time course studies revealed that the pro-apoptotic proteolytic activation of PKC δ occurs well before apoptotic cell death, and that cells resist early oxidative damage, suggesting that some key intrinsic compensatory responses protect neurons from the initial oxidative insult. Therefore, we speculated that the persistently active catalytic fragment of PKC δ might have other functions during the early stages of oxidative stress, and so we further explored downstream signaling mechanisms.

Protein kinase D1 (PKD1) is a calcium/calmodulin-dependent member of the CAMK kinase family and can be activated by dual phosphorylation of serine residues (Ser 744/748) in the catalytic domain by different PKCs, depending upon the cellular type and stimuli [22-24]. PKD1 is activated in response to multiple stimuli including growth factors, phorbol esters, G-protein coupled receptors, genotoxic stress and oxidative stress [25-28]. In non-neuronal cells, PKD1 activation has been shown to play a role in diverse cellular functions including proliferation, cytoskeletal reorganization, golgi function and immune response [27,29-32]. PKD1 has been shown to regulate various cell signaling molecules and pathways including ERK1/2, JNK pathways [33-35], effector enzymes like MnSOD that scavenge ROS [31,36], transcriptional regulators including NF- κ B and MEF2 [37,38], stress responsive chaperones like HSP27 [39], and some members of the HDAC family [26,31,40]. Recently, PKD1 was recognized as an important mitochondrial ROS sensor that translocates to the nucleus to switch on cell survival mechanisms [36]. Also, PKD1 activation loop phosphorylation has been shown as an

early marker of sympathetic neuronal DNA damage [41]. In neuronal models, PKD1 regulates trafficking of dendritic membrane proteins to maintain neuronal polarity and synaptic plasticity [42].

While many biological functions of PKD1 are beginning to emerge, the role of PKD1 in the brain, specifically in the nigral dopaminergic system, remains unknown. The relationship between PKD1 signaling and neurodegeneration has not yet been examined in a single study. Herein, we demonstrate that PKD1 closely interacts with PKC δ and serves as a key compensatory protective mechanism in dopaminergic neuronal cells during the early stages of oxidative insult.

Results

Role of PKC δ cleavage in the early stages of H₂O₂-induced oxidative stress in dopaminergic neuronal cells

H₂O₂ is a common oxidative insult used to probe various cellular signaling pathways in different cell types including neuronal cells [15,43,44]. We have already demonstrated that H₂O₂ causes dose- and time-dependent cytotoxicity, DNA fragmentation and cell death in the dopaminergic neuronal N27 cell model [15]. In order to determine the interrelationship between PKC δ proteolytic cleavage and oxidative stress-induced cell death, we examined the time course of PKC δ proteolytic activation and cell death. As shown in Figure 1A, generation of ROS occurs as early as 1 h after H₂O₂ treatment. Cytotoxicity begins between 90 and 120 min (Figure 1B). PKC δ proteolytic cleavage and the kinase activity increases during the early stage of H₂O₂ exposure at 60 min (Figure 1C, D). Comparison of PKC δ proteolytic activation and cytotoxicity at 60 min revealed that PKC δ proteolytic activation occurs during the early stages of oxidative stress preceding cell death (Figure 1E). There was no cell death during this intermediate period between ROS generation and PKC δ proteolytic activation. PKC δ knockdown by RNAi almost completely blocked the cell death induced by H₂O₂ at 120 min of treatment, demonstrating the pro-apoptotic function performed by PKC δ in N27 cells (Figure 1F). Importantly, a significant lag period between induction of cell death and PKC δ proteolytic activation was observed. This interesting observation prompted a search for cell signaling mechanisms associated with a compensatory protective response involving PKC δ proteolytic activation during early stages of oxidative stress.

Oxidative stress induces phosphorylation and activation of PKD1 in a time-dependent manner

We hypothesized that the proteolytically cleaved PKC δ might activate other downstream cell survival signaling molecules to counteract the early stage of oxidative

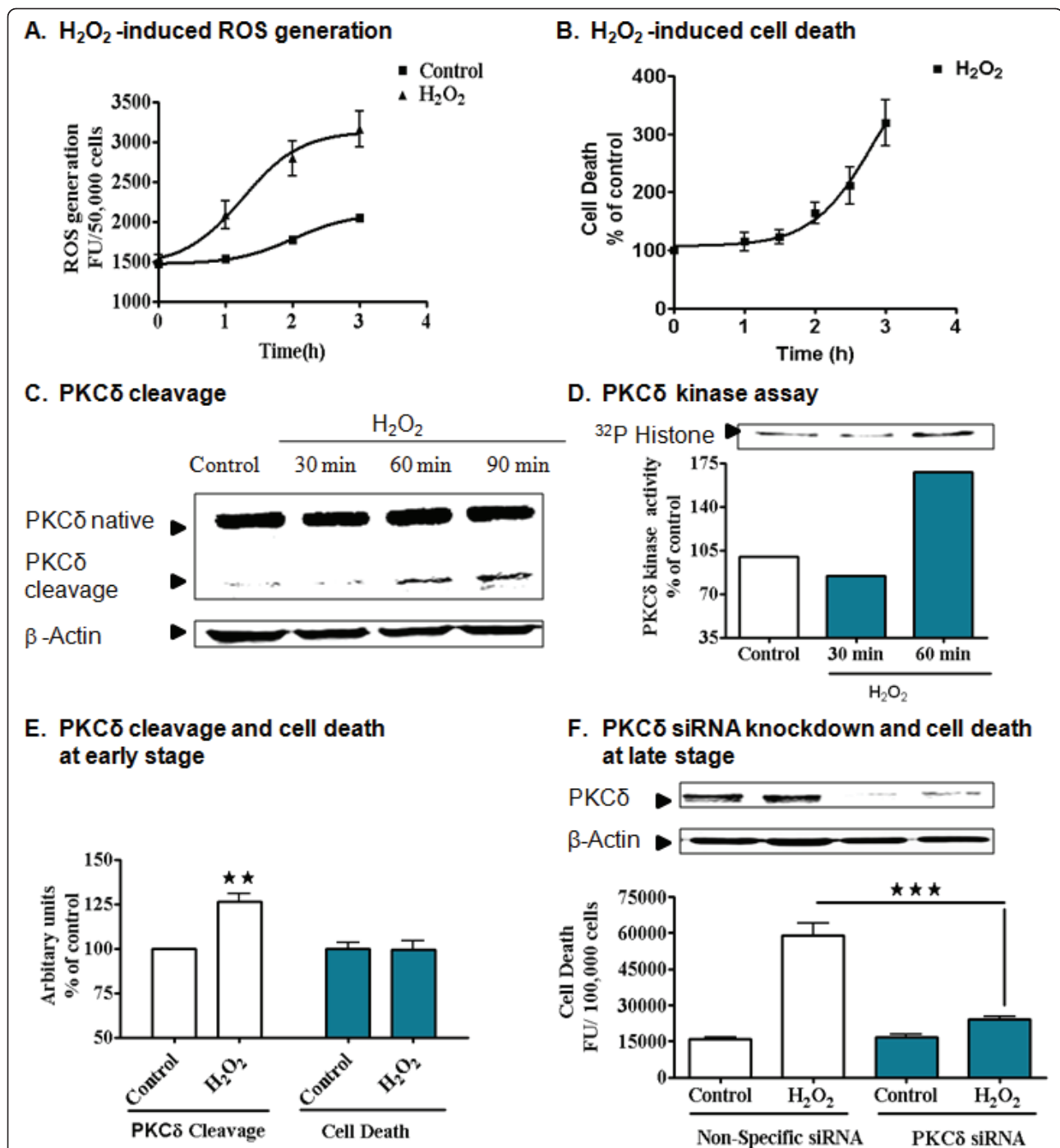
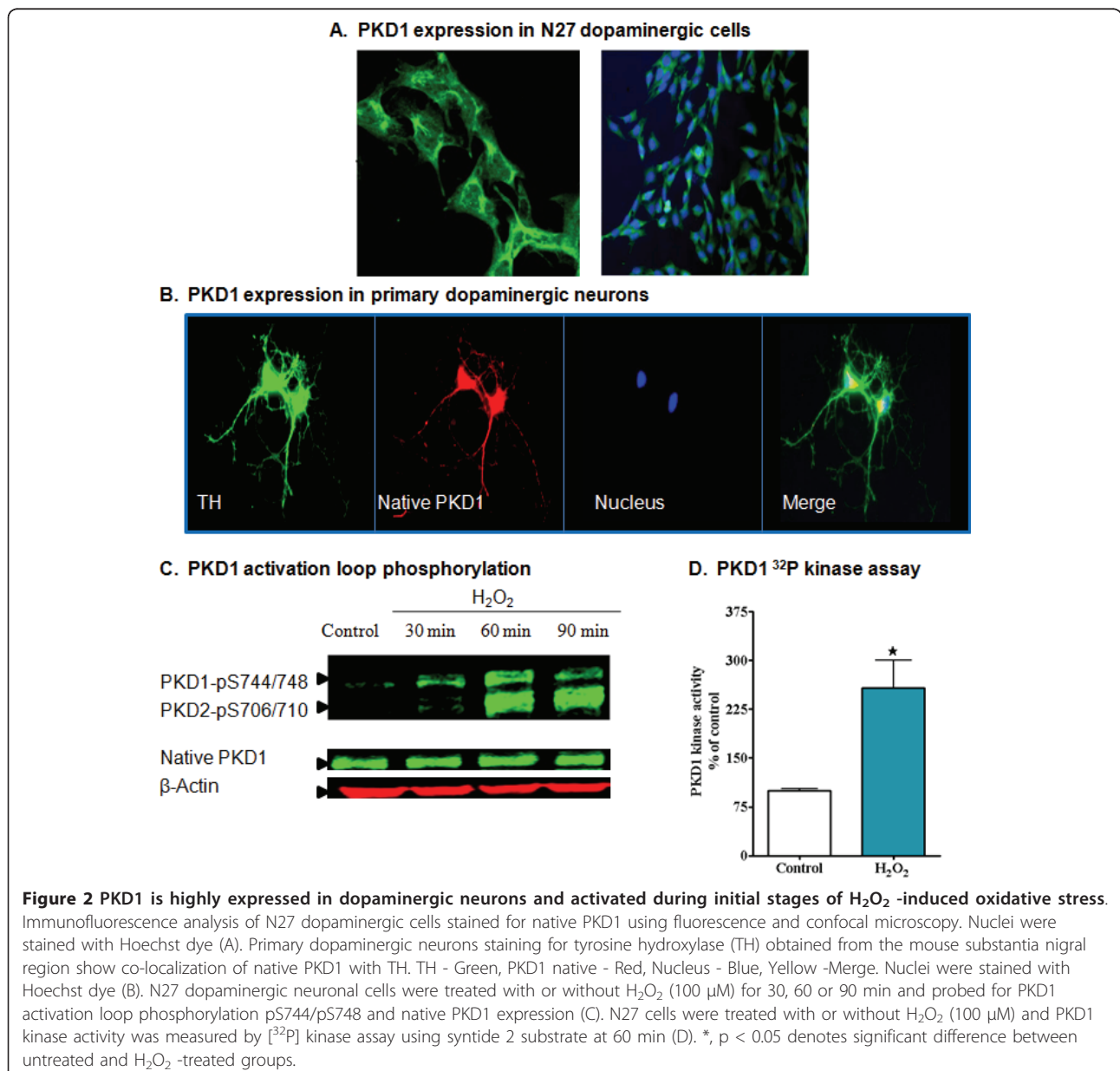


Figure 1 Relationship between PKCδ proteolytic activation and cell death during initial stages of H₂O₂-induced oxidative stress in N27 dopaminergic neuronal cell model. N27 dopaminergic cells were treated with H₂O₂ (100 μM) for 0-180 min and assayed for ROS generation using DCFDA dye (A) and for cytotoxicity using Sytox green dye (B). Non-linear regression graph from two or more independent experiments (n = 6-8). PKCδ cleavage was monitored by Western blot in a time dependent manner for 0 - 90 min in N27 dopaminergic cells treated with H₂O₂ (100 μM) (C). N27 dopaminergic cells were treated with H₂O₂ (100 μM) for 0, 30 or 60 minutes and PKCδ kinase activity was measured using [³²P] kinase assay; the bands were quantified for the graph (D). Data quantified from B and C were used to generate a bar graph and were compared with cytotoxicity and PKCδ cleavage following H₂O₂ exposure for 60 min. **, p < 0.01 as indicated by two-way ANOVA analysis using Bonferroni post test (E). N27 cells were transfected with 1 μM PKCδ siRNA and non-specific siRNA for 24 h and treated with 100 μM H₂O₂ and monitored for cytotoxicity using Sytox green dye, which showed significant protection from oxidative stress. ***, p < 0.001 denotes significant difference between non-specific siRNA- H₂O₂ and PKCδ siRNA H₂O₂-treated groups from two or more independent experiments (n = 6-8). Statistics were performed by one-way ANOVA analysis using Bonferroni post test (F).

insult. To test this hypothesis, we first used a bioinformatic approach to search for a key pro-survival molecule that interacts with PKC δ . Scansite Motif Scanner software [45] predicted that out of all the PKCs, only PKC δ can phosphorylate the protein kinase D1 (PKD1) activation loop ser residue at high stringency search (additional file 1). Further literature review indicated that PKD1 is an oxidative stress-responsive kinase that can be activated by phosphorylation at the activation loop (S744/S748) in non-neuronal models [22-25,46]. This encouraged us to look further for the expression and activation of PKD1 in dopaminergic neuronal cells. Immunocytochemical staining showed abundant

expression of PKD1 in N27 dopaminergic cells, as visualized by confocal and fluorescence microscopy (Figure 2A), which is similar to the expression pattern of PKC δ in this cell type. Importantly, PKD1 is also highly expressed in primary tyrosine hydroxylase (TH) positive dopaminergic neurons obtained from mouse substantia nigra in the cytosolic region (Figure 2B).

To determine if oxidative stress can induce PKD1 Ser 744/Ser 748 phosphorylation in the activation loop, we examined the ability of H₂O₂ to induce time-dependent PKD1 activation loop phosphorylation in N27 dopaminergic cells. As shown in Figure 2C, 100 μ M H₂O₂ induced transient PKD1 activation loop phosphorylation



corresponding to the 120 kDa band starting at around 30 min, peaking at 60 min and returning to control levels after 90 min, with native protein levels remaining the same. We also observed a second band around 100 kDa, which might correspond to the other isoform, PKD2. According to the manufacturers (Cell Signaling Technology), the phospho-specific antibody can also detect PKD2 Ser 706/Ser 710 phosphorylation because of the conserved activation loop residues between PKD isoforms. However, the PKD2 activation loop phosphorylation does not follow the transient pattern of activation observed with PKD1 (Figure 2C). Furthermore, the activation loop phosphorylation of PKD1 increased the PKD1 kinase activity, as measured by a [³²P] kinase assay using Syntide 2 substrate (Figure 2D). Collectively, these results demonstrate that oxidative stress activates PKD1 at early stages through phosphorylation of the dual phospho sites pS744/pS748.

Oxidative stress-induced PKD1 activation depends on PKC δ

To further determine whether PKC δ regulates PKD1, we used both pharmacological and genetic approaches to suppress the PKC δ and then measured the level of PKD1 activation. Treatment of N27 cells with the PKC δ inhibitor rottlerin (1 μ M) completely suppressed H₂O₂-induced PKD1 activation (Figure 3A), suggesting a potential role of PKC δ in PKD1 activation. Next, we used the PKC δ -specific siRNA to knockdown PKC δ and then probed for PKC δ expression and PKD1 phosphorylation. PKC δ knockdown completely attenuated PKD1 activation loop phosphorylation except for the bottom band, corresponding to 100 kDa PKD2 (Figure 3B). Furthermore, PKD1 activity was measured by [³²P] kinase assay in PKC δ knockdown samples. The suppression in PKC δ expression completely attenuated PKD1 activation during H₂O₂-mediated oxidative stress, confirming that PKC δ is indeed involved in PKD1 activation (Figure 3C). To show the specificity of PKC δ in PKD1 activation in neuronal models, we used PKC α siRNA and then measured PKD1 activation loop phosphorylation (additional file 2). Knockdown of PKC α did not attenuate PKD1 activation loop phosphorylation, further demonstrating the specificity of PKC δ -mediated PKD1 phosphorylation in dopaminergic neuronal models.

The constitutively active catalytic fragment of PKC δ mediates PKD1 activation

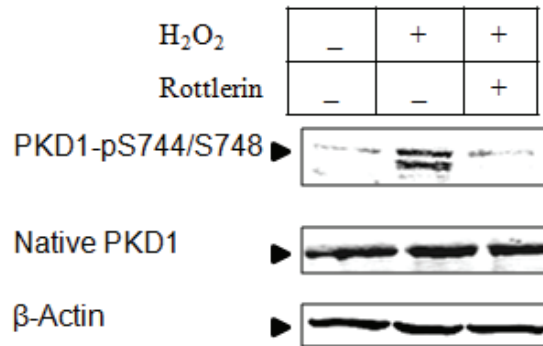
Time course analysis of PKC δ proteolytic cleavage and PKD1 activation revealed that the onset of proteolytic activation of PKC δ coincides with the maximal activation of PKD1 at 60 min following H₂O₂-induced oxidative stress (Figure 4A). Previously, we had shown that caspase-

3 inhibitor z-DEVD-fmk and pan caspase inhibitor ZVAD-fmk attenuate H₂O₂-induced proteolytic activation of PKC δ . Therefore, we examined whether the proteolytically activated PKC δ contributes to PKD1 activation. As shown in Figure 4B, co-treatment with z-DEVD-fmk and ZVAD-fmk for 1 h significantly blocked H₂O₂-induced activation loop phosphorylation of PKD1 as well as proteolytic cleavage of PKC δ . Coincidentally, H₂O₂-induced activation loop phosphorylation of PKD2 was not affected in the presence of these caspase inhibitors. To further confirm the role of proteolytically activated PKC δ in PKD1 activation, we used N27 cells stably expressing the PKC δ cleavage-resistant mutant PKC δ ^{D327A} (PKC δ -CRM). H₂O₂ treatment in PKC δ -CRM cells significantly attenuated PKD1 phosphorylation, confirming that the proteolytically cleaved PKC δ contributes to PKD1 phosphorylation during early stages of oxidative stress (Figure 4C). To further support this observation, we separately over-expressed the PKC δ catalytic fragment (V5-PKC δ -CF) and PKC δ regulatory fragment (V5-PKC δ -RF) using a lentiviral vector (plenti6/V5-D-TOPO) in N27 cells and then evaluated for activation of PKD1. Interestingly, V5-PKC δ -CF over-expressing cells had increased PKD1 phosphorylation, while V5-PKC δ -RF over-expressing cells did not increase phospho-PKD1 levels (Figure 4D). Collectively, these results demonstrate that the constitutively active catalytic fragment of PKC δ mediates PKD1 activation loop phosphorylation.

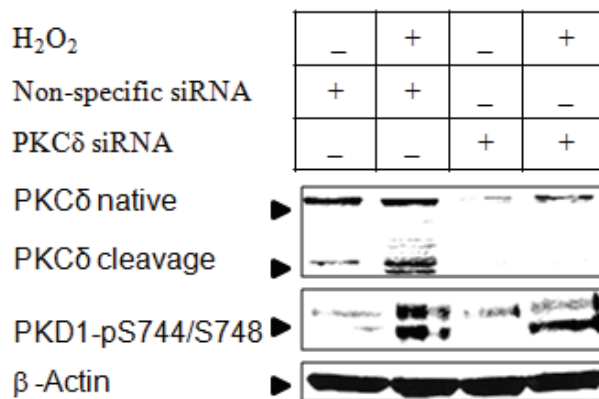
PKD1 activation functions as an anti-apoptotic protective mechanism against oxidative stress

We previously reported that proteolytically activated PKC δ (PKC δ -CF) mediates apoptosis in neurotoxicity models [15,19,47] and therefore, we initially hypothesized that PKC δ proteolytic cleavage-dependent activation of PKD1 may have a proapoptotic function. Surprisingly, PKD1 knockdown siRNA significantly augmented H₂O₂-induced cell death at 2 h, as measured by Sytox cell death assay (Figure 5A). PKD1 knockdown increased cell death by nearly twofold compared to control groups during H₂O₂ treatment, indicating a pro-survival role for PKD1 against oxidative stress (Figure 5B). Visualization of PKD1 knockdown cells by phase contrast and fluorescence microscopy further confirmed that PKD1 knockdown cells are more sensitive than non-specific control cells to H₂O₂-induced cytotoxicity (Figure 5C and 5D). We further measured the H₂O₂-induced apoptosis in PKD1 knockdown cells by DNA fragmentation ELISA assay at 3 h. The results showed an increase in DNA fragmentation in the PKD1 knockdown group, as compared to the non-specific siRNA control group, further confirming the anti-apoptotic role of PKD1 in dopaminergic cells (Figure 5E).

A. PKC δ dependent PKD1 activation



B. PKC δ siRNA knockdown and PKD1 activation



C. PKD1 ³²P kinase assay

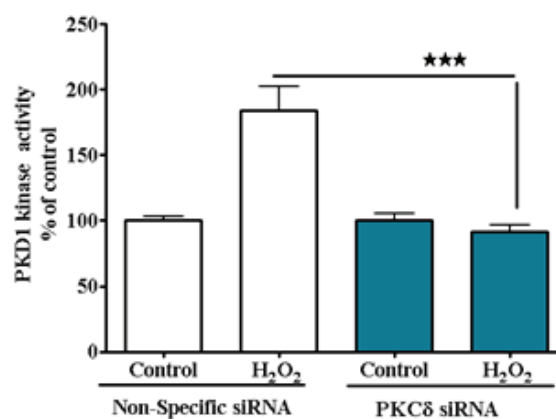
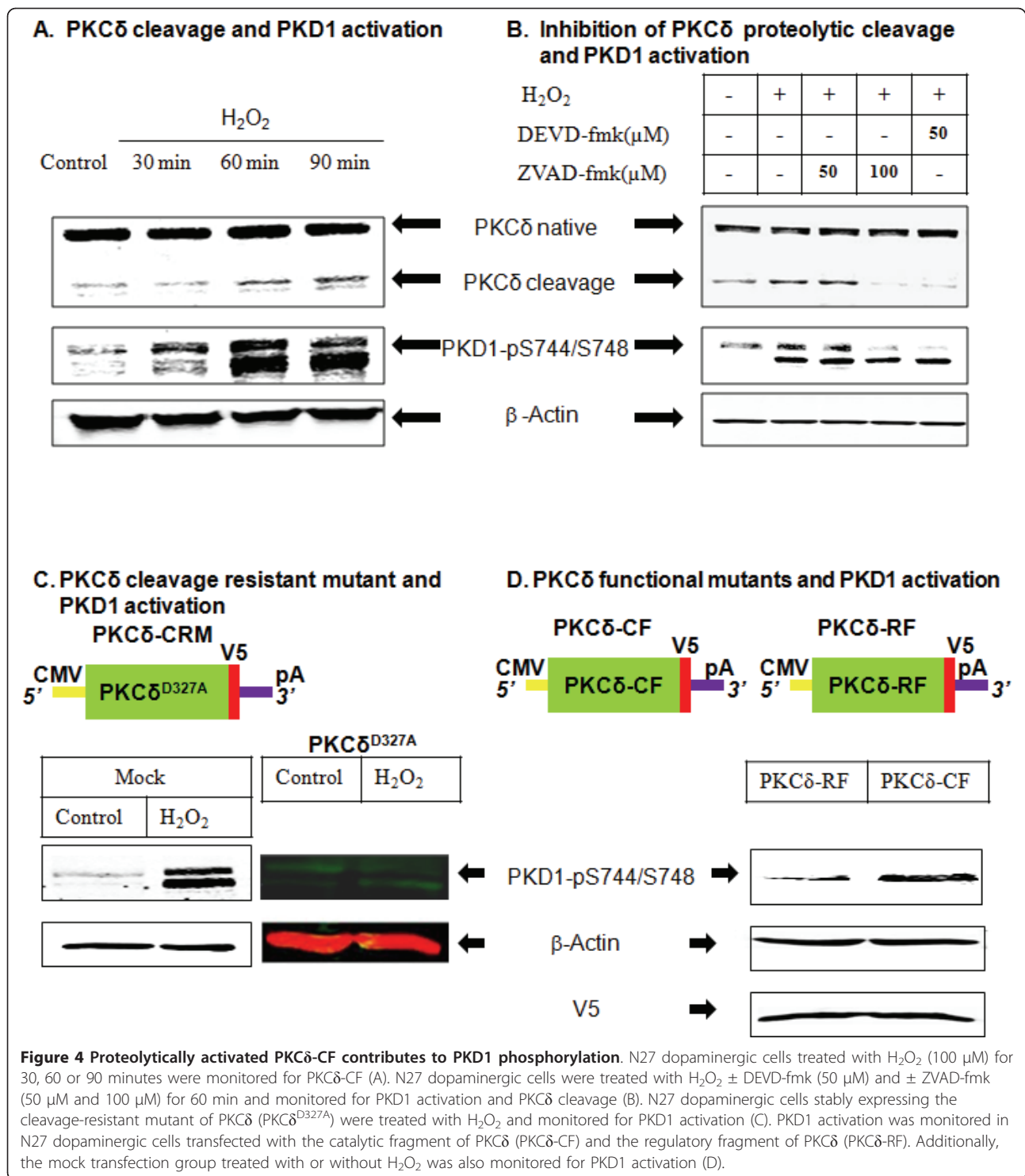


Figure 3 PKC δ dependent phosphorylation of PKD1 activation loop. N27 dopaminergic cells were treated with H₂O₂ (100 μ M) with or without 1 μ M rottlerin, and the lysates were probed for PKD1 activation phosphorylation (A). N27 dopaminergic cells were transfected with 1 μ M PKC δ siRNA and non-specific siRNA and monitored for PKC δ protein expression and PKD1 activation loop phosphorylation after H₂O₂ treatment (B) and PKD1 kinase activity assay was performed. **, p < 0.01 denotes significant difference between NS-siRNA-H₂O₂ and PKC δ -siRNA-H₂O₂ groups (C).

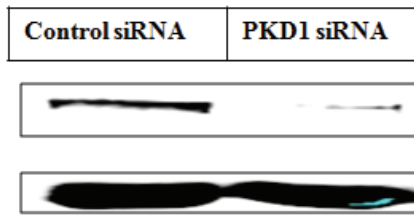


PKD1 C-terminal Ser 916 phosphorylation precedes PKD1 Ser 744/Ser 748 activation loop phosphorylation during oxidative stress

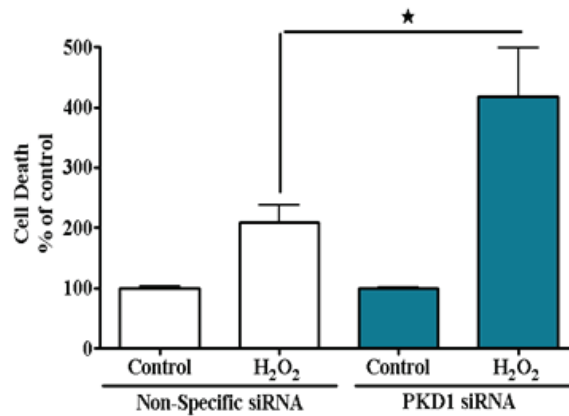
We also characterized the sequential mechanisms of PKD1 activation in dopaminergic neuronal cells. We investigated the other two key phosphorylation sites of PKD1, Tyr 469

and Ser 916. As shown in Figure 6A H₂O₂-induces transient phosphorylation of PKD1 Ser 916 in a time-dependent manner. The data suggests that PKD1 C-terminal Ser 916 was rapidly phosphorylated as early as 10 min after H₂O₂ treatment continues to increase up to 90 min and decreases at 150 min (Figure 6A). The phospho PKD1-

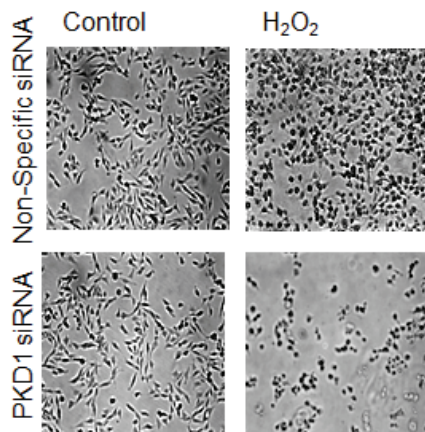
A. PKD1 siRNA knockdown



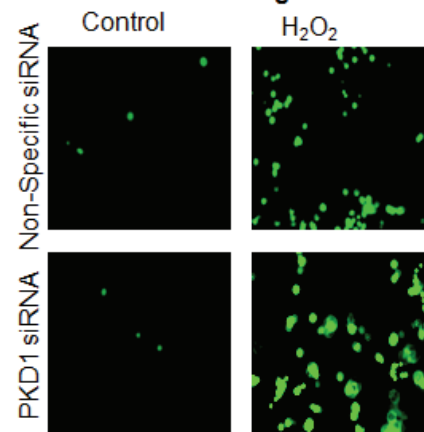
B. PKD1 siRNA knockdown and cell death



C. Phase-Contrast image



D. Fluorescence image



E. PKD1 siRNA knockdown and DNA fragmentation assay

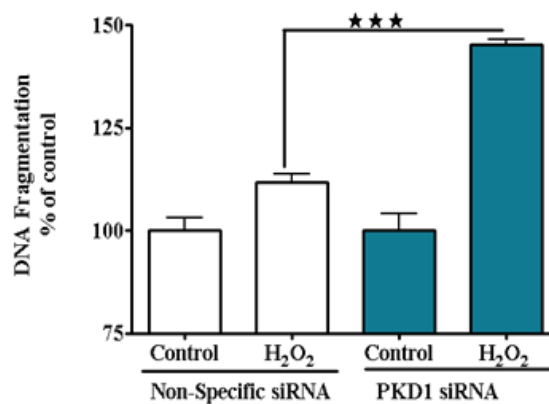
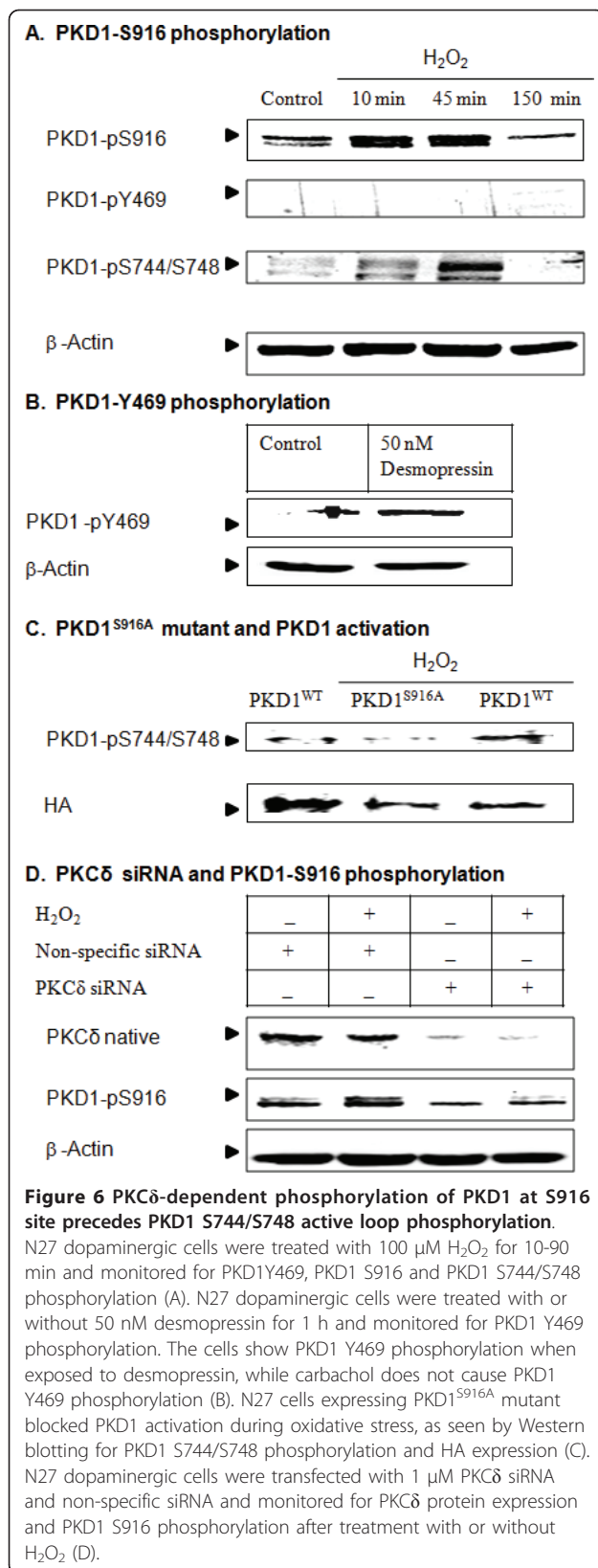


Figure 5 PKD1 has a cell survival function during oxidative stress in dopaminergic neuronal cells. N27 dopaminergic cells were transfected with 1 μ M PKD1 siRNA and non-specific siRNA (A) and treated with 100 μ M H₂O₂ for 120 minutes and monitored for cytotoxicity using Sytox green dye. Fluorescence measurements for the incorporation of Sytox green read using a fluorescence plate reader (B) and visualised by phase contrast and fluorescence microscopy (C&D). DNA fragmentation assay (E) showed increased cytotoxicity and apoptosis in PKD1 knocked down samples exposed to H₂O₂. *, p < 0.05 and ***, p < 0.001 denote significant difference between Non-Specific siRNA- H₂O₂ and PKD1 siRNA-H₂O₂ treated groups.

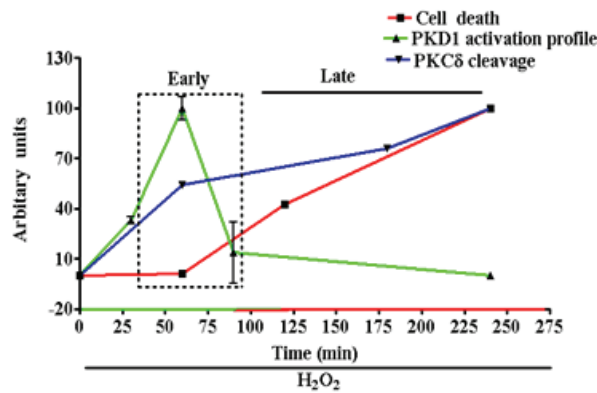


pS916 antibody detects a doublet PKD1 band. On the other hand, H₂O₂ failed to induce PKD1-tyr 469 phosphorylation in N27 dopaminergic cells (Figure 6A). Our results are consistent with a previous study suggesting that PKD1 tyr 469 phosphorylation does not occur in H₂O₂-treated Swiss 3T3 cells [48]. Similarly, it was shown that Vasopressin a GPCR agonist can activates PKD1 in Swiss 3T3 cells [30]. Since H₂O₂ did not phosphorylate tyr 469, we used desmopressin, a synthetic analogue of vasopressin to induce tyr 469 phosphorylation. Exposure of N27 dopaminergic cells to the positive control desmopressin induced tyr 469 phosphorylation (Figure 6B). To further evaluate the involvement of PKD1 Ser 916 phosphorylation in PKD1 activation, we overexpressed the PKD1^{S916A} mutant and PKD1^{WT} plasmids in dopaminergic cells and then stimulated the cells with H₂O₂. Overexpression of PKD1^{S916A} mutant blocked PKD1 activation loop phosphorylation (Figure 6C), suggesting that Ser 916 phosphorylation is an early event that has to occur prior to PKD1 activation loop phosphorylation. Furthermore, knockdown of PKCδ attenuated PKD1^{S916} phosphorylation, demonstrating that this is PKCδ dependent (Figure 6D). Together, these results suggest that PKD1 Ser 744/Ser 748 activation loop phosphorylation is intrinsically regulated by PKCδ via C-terminal phosphorylation of PKD1 Ser916.

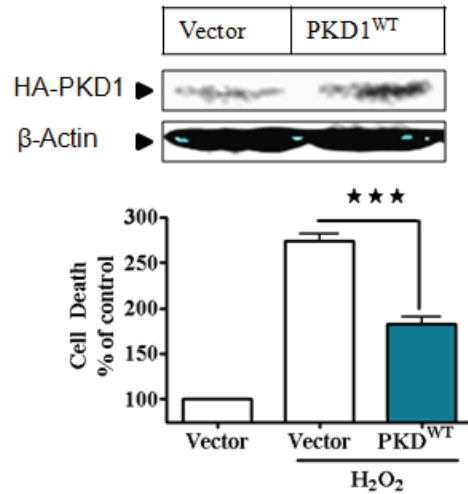
PKD1 activation acts as a protective compensatory mechanism during early stages of oxidative stress

To understand the relationship between PKCδ/PKD1 activation and neuronal cell death during oxidative stress, we quantified the PKCδ/PKD1 activation profile from the Western blots and then compared them with the profile of neuronal cell death during H₂O₂-induced oxidative stress. As shown in Figure 7A, PKD1 was activated at the early stages of oxidative insult and no measurable cell death was observed until PKD1 activation started declining to basal levels. Alternatively, PKCδ activation was concomitantly increased along with H₂O₂-induced cytotoxic cell death at the later stage of oxidative stress. Thus, the inverse correlation of PKD1 activation with cytotoxicity suggests that PKD1 activation may act as a compensatory protective response during early stages of oxidative insult. To test this hypothesis, we first overexpressed the full-length human PKD1 (PKD1^{WT}) in N27 cells and then examined the H₂O₂-induced cytotoxicity. As anticipated, PKD1^{WT} overexpression protected the dopaminergic cells against oxidative stress-induced cytotoxicity at 2 h (Figure 7B). In order to establish the pro-survival function of PKD1 activation, we examined whether overexpression of the PKD1^{S916A} phosphorylation defective mutant exacerbated H₂O₂-induced neuronal cell death. As shown in

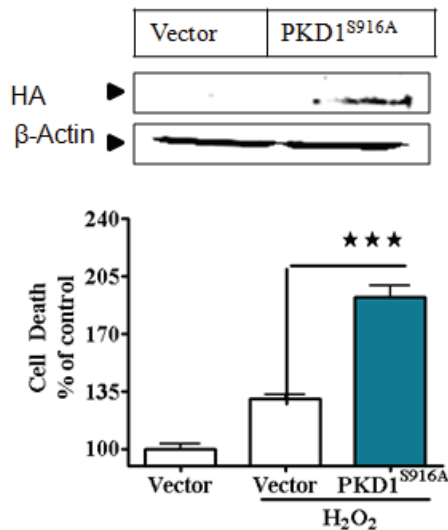
A. Activation profiles of PKD1, PKC δ and cell death



B. Cell survival in PKD1^{WT} expressing cells



C. Cell death in PKD1^{S916A} mutant



D. Cell survival in PKD1^{S744E/S748E} mutant

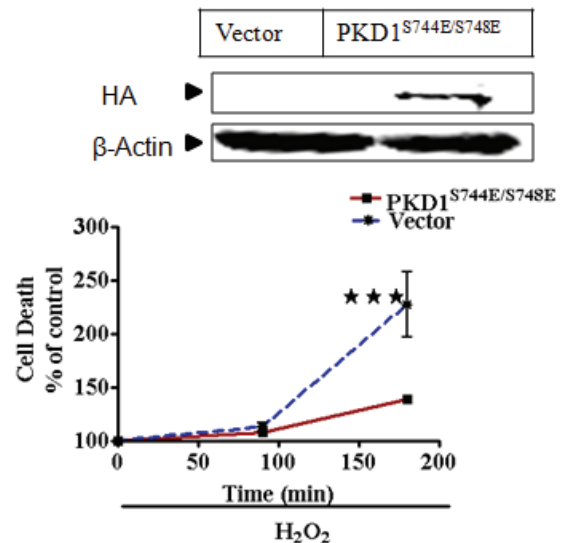


Figure 7 PKD1 activation acts as an early protective compensatory mechanism. A comparative time course graph based on quantifying PKD1 activation profile, PKC δ cleavage profile and cytotoxicity during H₂O₂ exposure (A). N27 dopaminergic cells transiently transfected with 5 μ M full length PKD1 plasmid (PKD1^{WT}) and 5 μ M vector plasmid were treated with or without 100 μ M H₂O₂ for 150 minutes and monitored for cytotoxicity using sytox green; PKD1 protected against cytotoxicity (B). N27 dopaminergic cells transiently transfected with 5 μ M PKD1^{S916A} plasmid and 5 μ M vector plasmid were treated with or without 100 μ M H₂O₂ for 150 minutes and monitored for cytotoxicity using sytox green; increased cytotoxicity was observed in the cells (C). N27 dopaminergic cells transiently transfected with 5 μ M PKD1^{S744E/S748E} and 5 μ M vector plasmid (D) were treated with or without 100 μ M H₂O₂ and monitored for cytotoxicity at various time points using sytox green. ***, p < 0.001 denotes significant difference between treatment groups from n \geq 6.

Figure 7C, overexpression of the PKD1^{S916A} phosphorylation defective mutant exacerbated H₂O₂-induced cytotoxic cell death as early as 90 minutes, compared to vector overexpressing cells. To further confirm our hypothesis that PKD1 activation loop phosphorylation acts as an early protective compensatory response, we overexpressed the activation loop active plasmid (PKD1^{S744E/S748E}), where the replacement of serine with

glutamate makes the kinase constitutively active [49], and cytotoxicity was monitored for up to 3h following H₂O₂ treatment (Figure 7D). The constitutively active PKD1 mutants tremendously suppressed the cytotoxicity, even during late stages of oxidative insult, indicating that PKD1 activation is a very significant early protective compensatory mechanism in dopaminergic cells. Collectively, these results demonstrate that PKD1

is a cell survival kinase that is activated during the early stages of oxidative stress to protect against cytotoxicity in dopaminergic cell models.

Activated PKD1 translocates to nucleus during oxidative stress in cell culture models of neurodegeneration

We also performed immunocytochemical staining to examine the subcellular localization of activated PKD1 during oxidative stress. Activated PKD1 (PKD1pS744/

pS748) (green) co-localized with the nuclear Hoechst stain (blue) at 1 h during H₂O₂-induced oxidative stress in N27 cells, as visualized by fluorescence microscopy (Figure 8A). These results indicate that activated PKD1 translocates to the nucleus of dopaminergic neuronal cells to carry out cell repair and survival functions.

Furthermore, we examined oxidative stress-induced PKD1 activation in primary mesencephalic dopaminergic neurons. Mouse primary mesencephalic neuronal

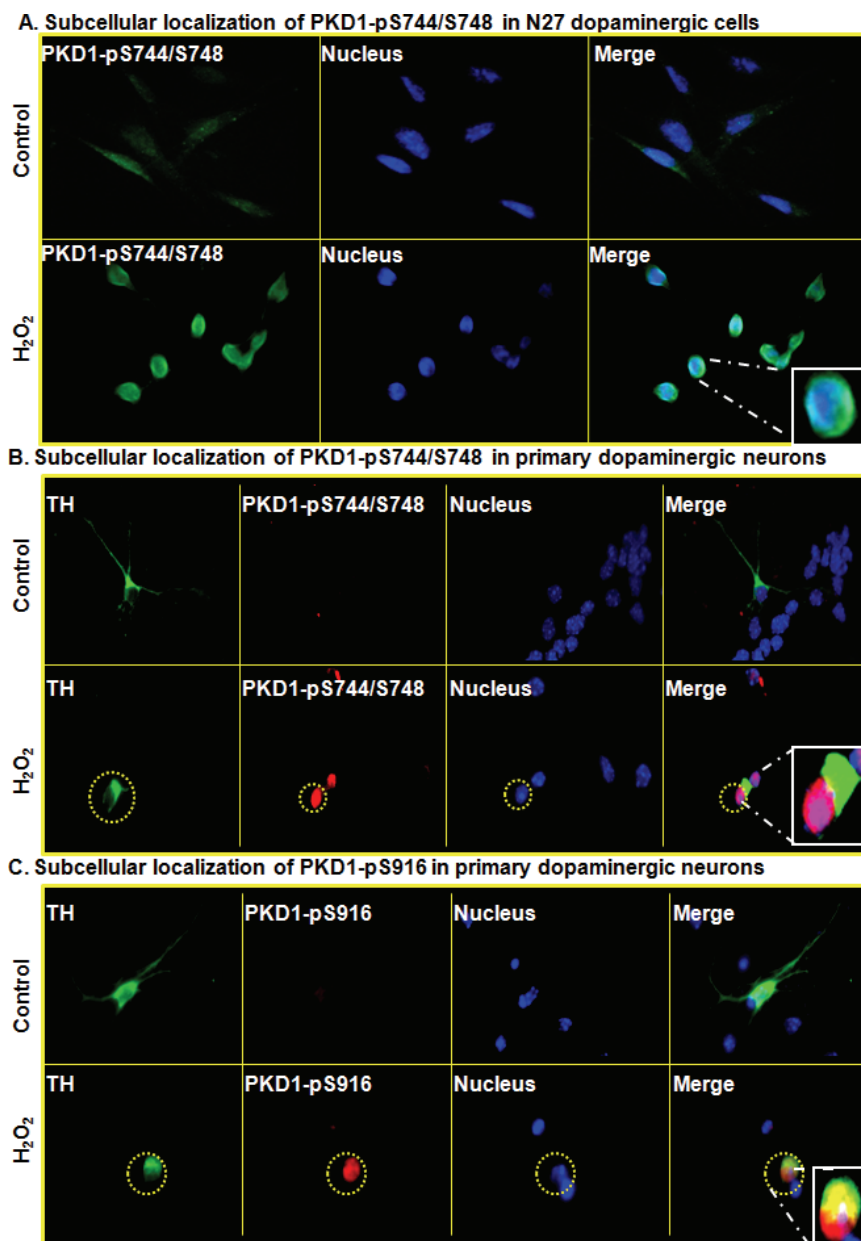


Figure 8 Activated PKD1 translocates to nucleus during H₂O₂-induced oxidative stress. Immunofluorescence analysis of N27 dopaminergic cells stained for activated PKD1 using fluorescence microscopy during H₂O₂ exposure show translocation to nucleus. PKD1pS744/S748 - Green, Nucleus - Blue. Nuclei were stained with Hoechst dye (A). Immunofluorescence analysis of primary dopaminergic neurons staining for TH obtained from the mouse substantia nigral region shows translocation of activated PKD1 to the nucleus during H₂O₂ exposure. TH-Green, PKD1pS744/S748 - Red, Nucleus - Blue, Merge - Pink. Nuclei were stained with Hoechst dye (B). Primary dopaminergic neurons staining for TH show presence of PKD1pS916 in both cytosol and nucleus during H₂O₂ exposure. TH - Green, PKD1pS916- Red, Nucleus - Blue, Pink - Merge in nucleus, yellow -Merge in cytosol. Nuclei were stained with Hoechst dye (C).

cultures were treated with a low dose of 10 μ M H₂O₂ to induce oxidative stress and then subcellular localization of PKD1 activation was monitored by TH/PKD1 double immunolabeling. Activated PKD1 (red) (PKD1 pS744/pS748) co-localized (pink) with the nuclear Hoechst stain (blue) following H₂O₂ treatment in primary mesencephalic neurons staining for TH (green), as visualized by fluorescence microscopy (Figure 8B). We also examined the activation profile of PKD1 pS916 C-terminal phosphorylation. PKD1pS916 (red) was localized (pink/yellow) in both cytosol and nucleus of TH +ve primary mesencephalic neurons staining for green during H₂O₂ -induced oxidative stress (Figure 8C).

Parkinsonian-specific toxicant causes PKD1 activation

Activation of this signaling pathway was also tested using the parkinsonian-specific toxicant 6-OHDA. We treated N27 cells with 100 μ M 6-OHDA and performed a time-course analysis for 1, 3, and 6 h. PKD1 activation started as early as 1 h and continued until 3 h before reaching control levels at around 6 h (Figure 9). This further confirmed the involvement of PKD1 signaling during parkinsonian-specific oxidative insult.

Discussion

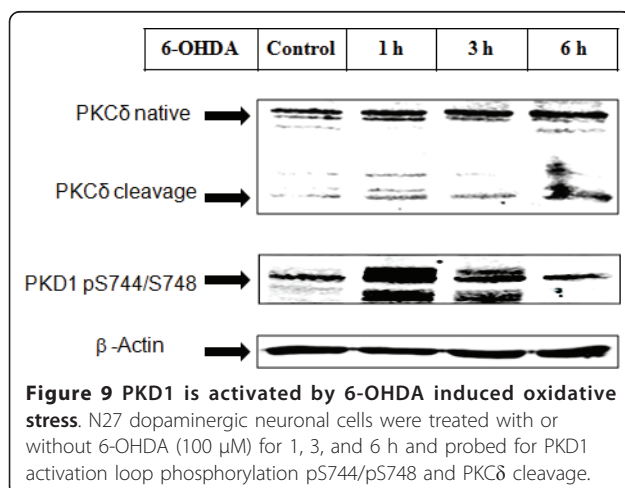
The present study reveals a novel protective compensatory signaling mechanism via PKC δ -PKD1 molecular interaction in dopaminergic neuronal cells. Through our collective results, we report for the first time four key findings in a dopaminergic neuronal model pertinent to oxidative stress-mediated neurodegenerative processes: (i) A proteolytically activated catalytic PKC δ fragment (PKC δ -CF) phosphorylates and activates protein kinase D1 (PKD1); (ii) PKD1 activation counteracts early stage oxidative damage and protects dopaminergic neuronal cells from cytotoxicity; (iii) PKC δ -dependent phosphorylation of ser 916 residue precedes ser 744/ser 748; (iv)

PKC δ - PKD1 crosstalk tightly regulates cell survival and cell death to maintain cellular homeostasis in response to oxidative damage. The elucidation of this compensatory signal transduction mechanism in neurodegenerative diseases may enhance understanding of degenerative processes and lead to development of novel treatment modalities.

H₂O₂-induced cytotoxicity causes apoptosis in neuronal and non-neuronal cells [15,43,44,50]. Generally, oxidative stress-induced apoptosis can be classified into early and late stages. DNA fragmentation occurs in the late stage of apoptosis and is preceded by ROS generation, mitochondrial dysfunction and caspase-3 activation and membrane phosphatidyl exposure [10,47]. In neurodegenerative disorders, especially PD, the signaling mechanisms that contribute to increased vulnerability of dopaminergic neurons to oxidative damage are still under investigation. Most current research focuses on cell death mechanisms in dopaminergic neurons. Some of the signaling kinases responsible for cell death mechanisms in PD include JNK, MLK, MAPK, LRRK2, etc. [51-54]. Earlier, the involvement of a novel biochemical mechanism for cell death in dopaminergic neurons through caspase-mediated proteolytic activation of PKC δ was demonstrated [15-19]. The high levels of persistently active PKC δ catalytic fragment mediate apoptosis during oxidative stress in both cell culture and animal models of PD [15-19]. We also have shown in our earlier study that a positive feedback loop exists during the late stages of oxidative stress, where the persistently active PKC δ catalytic fragment translocates to the mitochondria to promote cytochrome C release and apoptosis [16,17,55].

We previously demonstrated proteolytic activation of PKC δ occurs during the early stages of oxidative stress, even before cell death can occur, and coincides with the initiation of mitochondrial ROS generation/caspase-3 activation in dopaminergic neurons [15,17]. Thus, we speculated that proteolytically activated PKC δ might play a regulatory role during the early stages of apoptosis. Previous research suggests the presence of a variety of protective compensatory mechanisms that counteract the early oxidative insult [8-10]. Since we observed in our present study a significant lag time before induction of cell death during the early stages of oxidative stress (Figure 1B), we hypothesize that proteolytically activated PKC δ might sense the extent of oxidative damage and act as a homeostatic regulator in response to oxidative stress, modulating cell survival and cell death mechanisms through interactions with protective signaling molecules.

Protein kinase D1 (PKD1) is emerging as an important signaling molecule associated with oxidative stress in non-neuronal cell lines [31,35,36]. Studies have shown that oxidative stress increases PKD1 activation loop



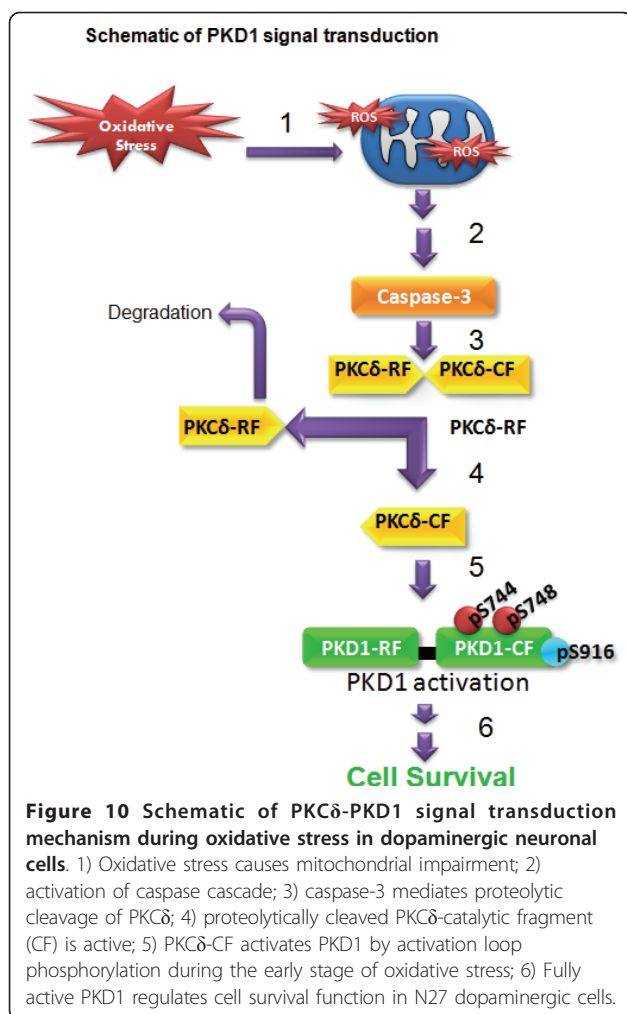
phosphorylation (pS744/pS748) via full length PKCs, including PKC δ , in non-neuronal models [37,56-59]. However, the functions of PKD1 during oxidative stress-induced neurodegeneration have not been studied previously. In the present study, we report that cleaved active PKC δ phosphorylates the activation loop of PKD1 and activates the kinase during the early stages of H₂O₂-induced oxidative stress in dopaminergic neuronal cells. We also observed a similar activation pattern for PKD1 and PKC δ during oxidative stress caused by the parkinsonian-specific toxicant 6-OHDA (Figure 9). To our knowledge, this is the first report of a novel cell survival/cell death signal regulation by the cleaved catalytic fragment of PKC δ at two different stages of apoptosis based on the extent of oxidative damage.

PKD1 is mainly activated by a diacylglycerol-dependent PKCs mechanism [22,60] or by PKD1 cleavage [61-63]. A recent study shows that PKD1 auto-inhibition is released through phosphorylation at the Y463 site in the regulatory domain, leading to the activation loop phosphorylation by PKC δ full length (PKC δ -FL) in Hela cells [24]. PKD1 is in a closed conformation during the resting stage, with the regulatory fragment having an autoinhibitory effect on the catalytic fragment [46,64]. Multiple phosphorylation sites on PKD1 seem to be important for its activation loop phosphorylation, depending on the cell types and stimuli. In human cancer cell lines, PKD1 can be phosphorylated at multiple sites including Y463, S910 (corresponding to murine Y469, S916) [24,65]. Phosphorylation of Ser 916 (murine) autophosphorylation site correlated with PKD1 activation loop phosphorylation [58,66]. During oxidative stress in non-neuronal models, Tyr 469 is phosphorylated by upstream kinases, which results in release of the Pleckstrin homology (PH) domain auto-inhibition prior to activation loop phosphorylation; this mechanism does not involve C-terminus Ser 916 phosphorylation [24,37]. In our dopaminergic neuronal models, oxidative stress failed to induce PKD1 Tyr 469 phosphorylation (Figure 6A), whereas PKD1 Tyr 469 phosphorylation was induced by the positive control desmopressin (Figure 6B). Our results demonstrate that the mechanism of PKD1 activation in dopaminergic neurons is distinct from the mechanisms in other non-neuronal models. We demonstrate that S916 phosphorylation, but not Tyr 469 phosphorylation, is a preceding event that occurs and is required for PKD1^{ser744/Ser748} activation loop phosphorylation (Figure 6). Our data suggest that Ser 916 phosphorylation on the C-terminal of PKD1 may open the conformation for full activation of the kinase through activation loop phosphorylation during oxidative stress in dopaminergic neurons. A detailed comparative analysis of PKC δ proteolytic activation, PKD1 activation

loop phosphorylation and the extent of cell death during oxidative stress revealed an interesting functional relationship between activation of kinases and regulation of cell death. Comparison of PKD1 activation and cytotoxicity shows that PKD1 activation is maximal during the early oxidative stress stage when no measurable cytotoxicity is noted (Figure 7A). Interestingly, when PKD1 activation begins to decline at the end of the early stage, cell death begins to occur. Also, the level of PKC δ proteolytic activation directly correlates with the extent of cell death at the later stage of oxidative stress. When the constitutively active PKD1 mutant (PKD1^{S744E/S748E}) is overexpressed, dopaminergic cells are resistant to H₂O₂-induced neurotoxicity, even during the late stages of oxidative stress (Figure 7D), which is consistent with our hypothesis that PKD1 activation protects against oxidative damage. The downstream signaling mechanisms of PKD1 activation in dopaminergic neuronal cells are not known. PKD1 translocates to the nucleus and regulates phosphorylation of HDACs and various transcription factors in various non-neuronal cell lines including B cell, cardiomyocytes & oestoblasts [31,23,40,67]. PKD1 translocation to the nucleus after activation in dopaminergic neurons is also noted in the present study (Figure 8), suggesting that nuclear translocation of PKD1 may activate key cell survival transcription factors and genes. Thus, we suggest that PKD1 functions as a cell survival switch and turns 'ON' a protective compensatory mechanism in dopaminergic neurons. Studies are underway to characterize the downstream protective response of PKD1 signaling in nigral dopaminergic neurons.

Conclusions

Our results demonstrate that the PKD1-mediated protective mechanism is a novel signal transduction pathway that regulates cell survival and cell death during various stages of oxidative stress in dopaminergic neuronal cells. As depicted in Figure 10, in the early stages of oxidative insult, PKC δ acts as an oxidative stress sensor/regulator and activates PKD1, which serves as a key compensatory protective mechanism against oxidative damage. However, prolonged oxidative insult creates a homeostatic imbalance, causing deactivation of PKD1 and persistent proteolytic activation of PKC δ that contribute to extensive neuronal damage. We previously showed a parallel proapoptotic mechanism involving translocation of PKC δ catalytic fragments to the mitochondria, resulting in a persistent increase in caspase-3 dependent PKC δ proteolytic cleavage via a positive feedback loop mechanism [16,17,55]. Our results suggest that positive modulation of the PKD1-mediated protective mechanism against oxidative damage in



dopaminergic neurons may provide novel neuroprotective strategies for treatment of PD.

Materials and methods

Cell Culture

The immortalized rat mesencephalic dopaminergic neuronal cell line (N27) was a kind gift from Dr. Kedar N. Prasad (University of Colorado Health Sciences Center, Denver, CO). N27 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM l-glutamine, 50 units of penicillin, and 50 μ g/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C, as described previously [16]. N27 cells are used widely as a cell culture model for PD [20,15,21,16,68].

Primary mesencephalic neuronal culture

Primary mesencephalic neuronal cultures were prepared from the ventral mesencephalon of gestational 16- to 18-day-old mouse embryos, as described earlier [69]. Tissues were dissected from E16 to E18 mouse embryos

maintained in ice cold Ca²⁺-free Hanks' balanced salt solution and then dissociated in Hanks' balanced salt solution containing trypsin-0.25% EDTA for 30 min at 37°C. The dissociated cells were then plated at equal density (0.5 \times 10⁶ cells) on 12 mm coverslips precoated with 0.1 mg/ml poly-D-lysine. Cultures were maintained in neurobasal medium fortified with B-27 supplements, 500 μ M l-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). The cells were maintained in a humidified CO₂ incubator (5% CO₂ and 37°C). Half of the culture medium was replaced every 2 days. Approximately 6- to 7-day-old cultures were used for experiments. Primary mesencephalic dopaminergic neuronal cells were exposed to 10 μ M for 1 h.

Treatment Paradigm

N27 cells were exposed to H₂O₂ (100 μ M) for 0-4 h at 37°C. Primary neurons were exposed to H₂O₂ (10 μ M) for 1 h. Cell lysates were used for Western blotting and immunoprecipitation studies. Untreated cells were grown in the complete medium and used as the experimental control.

Cytotoxicity Assays

Cell death was determined using the Sytox green cytotoxicity assay, after exposing the N27 cells to H₂O₂ (100 μ M), as described previously. This cytotoxicity assay was optimized for a multiwell format, which is more efficient and sensitive than other cytotoxicity measurements [70,71]. Briefly, N27 cells were grown in 24-well cell culture plates at 100,000 cells per well and treated with H₂O₂ (100 μ M) and 1 μ M Sytox green fluorescent dye. The Sytox green assay allows dead cells to be viewed directly under a fluorescence microscope, as well as quantitatively measured with a fluorescence microplate reader (excitation 485 nm; emission 538 nm) (Biotek). Phase contrast and fluorescent images were taken after H₂O₂ exposure with a NIKON TE2000 microscope, and pictures were captured with a SPOT digital camera.

ROS Generation Assay

ROS generation was monitored by CM-DCFDA dye, as described previously [15,72]. This is a non-fluorescent dye in its reduced form, but after oxidation in the cells, the acetate group is removed by cellular esterases, resulting in fluorescence. N27 cells were seeded in 48-well plates at a confluence of 40,000 cells/well for 24 h. First, cells were loaded with 10 μ M CM-DCFDA dye (Invitrogen) at 37°C for 1 h in the dark. Cells were then treated with H₂O₂ in Hanks' balanced salt solution (HBSS) and the fluorescence of the cells was measured using the synergy 2 fluorescence plate reader (Biotek) at various time points (excitation 485 nm; emission 538 nm).

Immunocytochemistry

The primary mesencephalic neurons or N27 cells after H₂O₂ treatment were fixed with 4% paraformaldehyde and processed for immunocytochemical staining. First, nonspecific sites were blocked with 2% bovine serum albumin, 0.5% Triton and 0.05% Tween-20 in phosphate-buffered saline (PBS) for 20 min. The cells then were incubated with antibodies directed against TH, native PKD1 and PKD1-pS744/S748 in PBS containing 1% BSA at 4°C overnight, followed by incubation with Alexa 488 and Alexa 568 conjugated secondary antibodies in PBS containing 1% BSA. Secondary antibody treatments were followed by incubation with Hoechst 33342 dye for 5 min at room temperature to stain the nucleus. The coverslips containing stained cells were washed with PBS, mounted on slides, and viewed under a Nikon inverted fluorescence microscope (model TE-2000U; Nikon, Tokyo, Japan). Both fluorescence and confocal images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Western Blot Analysis

Cells were lysed in either modified RIPA buffer or M-PER buffer (Thermo Scientific) for Western blot, immunoprecipitation and kinase assays. Lysates containing equal amounts of protein were loaded in each lane and separated on 10-12% SDS-PAGE, as described previously (Kaul et al., 2003). PKD1 polyclonal (1:1000), PKC δ polyclonal (1:1000), PKD1-pS744/S748 (1:1000), PKD1-pS916 (1:1000), PKD1-pY469 (1:1000), - and β -actin (1:10000) antibodies were used to blot the membranes. IR dye-800 conjugated anti-rabbit (1:5000) and Alexa Fluor 680 conjugated anti-mouse (1:10000) were used for antibody detection with the Odyssey IR Imaging system (LICOR), as previously described.

PKC δ Kinase Assay

Immunoprecipitation and PKC δ kinase assay were performed as described earlier [15]. After cell lysis, cell were immunoprecipitated using a polyclonal PKC δ rabbit antibody and protein A Sepharose, and washed three times with PKC δ kinase buffer (40 mM Tris (pH 7.4), 20 mM MgCl₂, 20 μ M ATP, 2.5 mM CaCl₂). The reaction was started by adding 20 μ l of buffer containing 0.4 mg histone and 5 μ Ci of [γ -³²P] ATP (4,500 Ci/mM). After incubation for 10 min at 30°C, SDS loading buffer (2X) was added to the samples to terminate the reaction. The reaction products were separated on SDS-PAGE (12%), and the H1-phosphorylated bands were detected using a phosphoimager (Fujifilm FLA-5100) and quantified with MultiGauge V3.0 software.

Protein Kinase D1 Kinase Assay

The cells were exposed to H₂O₂ (100 μ M) for 1 h and cell lysates were immunoprecipitated, as previously reported, with native PKD1 antibody (Santa Cruz). The kinase reaction was carried out at room temperature for 20 min after adding 10 μ l of kinase substrate mix (0.1 mM ATP + 10 μ ci [γ -³²P] ATP + 2 μ g Syntide 2 peptide substrate in kinase buffer). Kinase buffer contains 20 mM Tris pH 7.5, 10 mM MgCl₂, and 1 mM DTT. The samples were centrifuged to terminate the kinase reaction, and the supernatants containing the phosphorylated peptide were applied as spots to P81 phosphocellulose squares (Whatmann). The papers were washed four times with 0.75% phosphoric acid and once with acetone and dried, and activity was determined by liquid scintillation counting. The samples were also loaded on a SDS-PAGE and probed for native PKD1 to determine equal loading.

DNA Fragmentation Assay

DNA fragmentation was measured using the Cell Death Detection ELISA Plus assay kit (Roche), for the detection of early apoptotic death, as described previously [15,72]. After 100 μ M H₂O₂ treatment, the cells were spun down at 200 \times g for 5 min and washed once with PBS. Then cells were lysed with lysis buffer provided with the kit. After lysis, the samples were spun down at 10,000 rpm for 10 min to collect the supernatant that was used to measure DNA fragmentation. The supernatants were further dispensed into the microtiter plates coated with streptavidin containing HRP-conjugated antibody cocktail that can detect the nucleosomes. After 2 h incubation, the HRP substrate provided in the kit was added. Measurements were taken in a Synergy 2 multiwell plate reader at 405 nm, with 490 nm as a reference reading.

Transient and Stable Transfections

cDNA encoding PKC δ catalytic fragment (PKC δ -CF), PKC δ regulatory fragment (PKC δ -RF) and PKC δ caspase-resistant mutant (PKC δ -CRM) (PKC δ ^{D327A}) from the pEGFPN1 vector were subcloned into the lentiviral expression vector plenti6/V5-d-TOPO in our lab (herein referred to as V5-PKC δ -CF, V5-PKC δ -RF, V5-PKC δ -CRM) [15,73]. ViraPower Lentiviral Expression System (Invitrogen) was used to establish stable transfections of a caspase-resistant mutant of PKC δ ^{D327A} [73]. Full-length human PKD1 plasmid (PKD1-FL), PKD1 activation loop, active PKD1^{S744E/S748E} (PKD1-CA) and PKD1^{S916A} mutants were obtained from Addgene, Inc. [74,49,37]. Electroporation was carried out with an Amaxa Nucleofector transfection instrument, as per the manufacturer's protocol. The transfected cells were then transferred to T-75 flasks or 6-well plates as desired and allowed to grow for a 24 h period before the treatment.

RNAi

PKC δ -siRNA was prepared by an in vitro transcription method, as described previously [20]. PKC δ -siRNA effectively suppressed > 80% of PKC δ protein expression levels within 24 h post-transfection. Predesigned PKD1-siRNA was purchased from IDT, Inc. PKD1-siRNA effectively suppressed > 80% of PKD1 protein expression levels after 36 h post-transfection. N27 cells (50-70% confluence) were transfected with siRNA duplexes using an Amaxa Nucleofector kit (Amaxa), as described in our previous study [20].

Statistical Analysis

Data analysis was performed using Prism 3.0 software (GraphPad Software, San Diego, CA). Bonferroni's multiple comparison testing was used to find the significant differences between treatment and control groups. Differences with $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered significantly different from $n \geq 6$ from two or more independent experiments, and are indicated in the figures.

Additional material

Additional file 1: Rat PKD1 amino acid sequence obtained from Swiss-Prot database (ID: Q9WTQ1) was analyzed using Scansite Motif software to identify the upstream PKCs that phosphorylate the PKD1 activation loop serine residues. The analysis done at high stringency shows that only PKC δ phosphorylates PKD1 at the activation loop residue Serine 744 site.

Additional file 2: N27 cells were transfected with 1 μ M PKC α siRNA and non-specific siRNA and monitored for PKC α protein expression and PKD1pS744/S748 after H₂O₂ treatment. PKC α knockdown did not cause attenuation in PKD1 activation loop phosphorylation.

Abbreviations

PD: Parkinson's disease; PKD1: Protein kinase D1; MAPK: Mitogen-activated protein kinases; PKC δ : Protein kinase C delta; CAMK: Ca²⁺/Calmodulin-Dependent Protein Kinase II; JNK: c-Jun N-terminal kinases; LRRK2: Leucine-rich repeat kinase 2 (LRRK2); MLK: Mixed-lineage kinase; ROS: Reactive oxygen species; MnSOD: Manganese superoxide dismutase; WB: Western Blot; PKC: Protein kinase C; PKC α : Protein kinase C alpha.

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Authors' contributions

All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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